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SWETA SARMAH

IDENTIFICAÇÃO DE NOVOS REGULADORES DA EXPRESSÃO DAF-16/FOXO

UNCOVERING THE NEW REGULATORS OF DAF-16/FOXO EXPRESSION

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Dissertação apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do Título de Mestra em Genética e Biologia Molecular, na área de Genética Animal e Evolução

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Orientador: MARCELO ALVES DA SILVA MORI

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Os membros da Comissão Examinadora acima assinaram a Ata de Defesa, que se encontra no processo de vida acadêmica do aluno.

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to the Journeyers to the East

to my Family

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RESUMO

A via de sinalização de insulina/IGF-1 (IIS) é central para o crescimento, o metabolismo e o envelhecimento em diferentes espécies. Em *C. elegans*, os principais componentes dessa via são o ortólogo do receptor de insulina/IGF-1 DAF-2 e o ortólogo do fator de transcrição FoxO DAF-16. Mutações no gene *daf-2* fazem os vermes viverem duas vezes mais, e esse fenômeno é totalmente dependente da ativação de DAF-16. Da mesma forma, outras condições que prolongam a vida de *C. elegans*, como a ablação da linha germinativa e certos protocolos de restrição alimentar, requerem DAF-16. Em uma varredura de RNAi, encontramos *adr-1*, *nrde-1*, *wago-1* e *set-25* como reguladores positivos da expressão e translocação do transgene repórter de DAF-16 em vermes sem linha germinativa. Esses genes estão envolvidos na modificação de histonas e/ou em vias de silenciamento gênico mediada por RNAs pequenos, e alguns participam da herança intergeracional. Isso sugere que o DAF-16 e o envelhecimento podem estar sujeitos à regulação epigenética por meio de mecanismos previamente não identificados. Neste projeto, confirmamos por vários métodos que DAF-16 é realmente controlado por essas proteínas, especialmente por NRDE-1. Também descobrimos que eles participam da regulação do tempo de vida e da formação de *dauer* mediada por DAF-16. Nossos dados de epistasia indicam que NRDE-1 interage com o AKT-1 para controlar o tempo de vida. Também foi possível demonstrar que NRDE-1 atua a jusante ou paralelo às quinases conhecidas por fosforilar e ativar AKT-1, como AGE- 1/PI3K e TORC2, sugerindo que NRDE-1 atua por meio de um mecanismo sem precedentes para regular a translocação nuclear de DAF-16. Finalmente, demonstramos que outros componentes do complexo NRDE (isto é, NRDE-2, NRDE-3 e, mais importante, NRDE-4) têm interações com a via de IIS. Propomos um novo mecanismo de regulação do tempo de vida de *C. elegans* por meio da interação entre proteínas envolvidas na maquinaria de RNAs endógenos pequenos e a via de IIS.

Palavras-chave: envelhecimento, *C. elegans*, regulação de genes, metabolism.

ABSTRACT

The insulin/IGF-1-like signalling (IIS) pathway is central to growth, metabolism and aging across the evolutionary spectrum. In *C. elegans*, key components of this pathway are the insulin/IGF-1 receptor ortholog DAF-2 and the FoxO transcription factor ortholog DAF-16. Mutations in the *daf-2* gene make worms live twice as long, and this phenomenon is entirely dependent on DAF-16 activation. Similarly, other conditions that extend lifespan in *C. elegans*, such as germline ablation and certain protocols of dietary restriction, require DAF-16. In a RNAi screen, we found *adr-1, nrde-1, wago-1,* and *set-25* as positive regulators of a DAF-16 reporter transgene expression in germline-less worms. These genes are involved in histone modification and/or small RNA-mediated silencing pathways, and some participate in transgenerational inheritance. This suggests that DAF-16 and aging may be subject to epigenetic regulation through such previously unidentified mechanisms. In this project we confirmed by several methods that DAF-16 is indeed controlled by these proteins, especially NRDE-1. We also found that they participate in DAF-16-mediated lifespan and *dauer* regulation. Our epistasis data indicate that NRDE-1 interacts with AKT-1 to control lifespan. We could also demonstrate that NRDE-1 acts downstream or in parallel to kinases known to phosphorylate and activate AKT-1, such as AGE- 1/PI3K and TORC2, suggesting that NRDE-1 acts through an unprecedented mechanism to regulate DAF-16 nuclear translocation. Finally, we demonstrate that other components of the NRDE complex (i.e., NRDE-2, NRDE-3 and more importantly NRDE-4) have interactions with the IIS pathway. We propose a new mechanism of lifespan regulation through the interaction between proteins involved in endogenous small RNA machinery and the IIS pathway.

Keywords: aging, *C. elegans*, gene regulation, metabolism.

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1. INTRODUCTION

1.1. Aging

Stars age as does the universe in which they live. The universe evolves from its primal state of intense heat to elementary nuclei, later to become atoms. The simplest atoms that come to exist around 380,000 years after the primal event often called the Big-Bang are hydrogen and helium. This uniform cloud of hydrogen and helium become stars and galaxies as it ages. Stars age, forming oxygen, nitrogen, carbon and so on till in the last pulsations they form iron and explode, scattering the stardust out of which we later emerge as biological beings. Aging and evolution remain the constant theme, whether it is of the universe or the beings and creations it gives birth to. For humans, the theme acquires a personal and societal dimension and thus understanding aging adds a scientific element to deep philosophical and social considerations.

Aging comes with a number of changes such as physiological, psychological, social and many others, leading to increased vulnerability to different diseases such as type 2 diabetes, cancer, hypertension, cardiovascular diseases, neurodegenerative disorders, etc. [1] and ultimately death.

The first demonstration that aging can be modified genetically was conducted using the free-living nematode *C. elegans*, in which mutations in the insulin/IGF-1 signalling (IIS) pathway resulted in doubling of the worm lifespan [2] [3] [4]. *C. elegans* is a primitive species that still retains many of the underlying biological characteristics that are fundamental to human biology. The lifespan of a *C. elegans*, as introduced in section 1.2. is so much smaller than that of a human that one can expect to device experiments on aging of this small being and hope to reach significant insights into aging at a human timescale and potentially lead to treatments targeted at preventing the development of age-related diseases and maintaining a healthier and prolonged human life.

1.2. *C. elegans* as a model organism

C. elegans is a small, transparent, microscopic soil nematode that feeds on microbes. Sydney Brenner first introduced it in 1974 as a model organism for genetic studies [5]. It is ~ 1mm in length and can be grown on Nematode Growth Medium (NGM) plates containing a lawn of bacteria [5]. They have a genome size of 100 Mb and 6 chromosomes. Genetic manipulations can be quickly done, and phenotypic changes can be well observed in them. Gene manipulation is generally done by feeding the *C. elegans* with bacteria expressing gene-specific dsRNA (RNAi) or creating transgenic lines overexpressing the chosen gene. Indeed, RNAi and transgene-mediated GFP expression have been first demonstrated in *C. elegans* and these discoveries rendered Nobel prizes in Physiology and Medicine and Chemistry, respectively [6] [7]. Worm strains can be frozen at -80°C or harvested and frozen in liquid nitrogen for long-term storage. The great advantage of *C. elegans*, especially in aging biology research, is their short lifespan; for wildtype animals, this is 18–20 days at 20°C [8] and it is to be noted that the development of *C. elegans* is temperature dependent.

C. elegans have a cylindrical body, mainly consisting of an outer tube (body wall) which is made up of the cuticle, hypodermis, excretory system, neurons, muscles; and an inner tube, which consists of the pharynx (responsible for the intake of food), intestine, and gonad [9]. They have two sexes; hermaphrodites (XX) and males (XO). The hermaphrodites are much more prevalent (>99%) and are known to have 959 somatic cells, out of which about 300 are considered to be neurons [10].

Males generally occur at a very low frequency and can easily be distinguished from hermaphrodites based on the tail morphology. Male larvae initially display the same body plan as hermaphrodites, but as their sexual organs begin to develop, the shape of their later half changes from stage L2 onwards [11] [12] [13], which then functions during mating (sperm transfer to the vulva of hermaphrodite)[14][15].

Males, generally used for outcrossing procedures, can be produced by mating with the hermaphrodites, either by normal segregation which gives rise to 50% hermaphrodites and 50% hermaphrodites or by spontaneous X chromosome nondisjunction events [16]. Also, *him* (high incidence males) mutants, especially *him-1, him-5* and *him-8* can be used for the production of a large number of males ~20-40% [17].

An adult self-fertilizing hermaphrodite can produce up to 300 individuals in a progeny, although if the mating occurs with a male, they can produce up to 1200-1400 progeny [18]. After hatching, the offspring develops rapidly through four larval stages, i.e., L1–L4 to adult in 3.5 days when maintained at 20°C, also known as the reproductive development. Typically, the hermaphrodites reproduce over a 3 to 5 days period and live for another 2–3 weeks.

1.3. *Dauers* and the genetics behind it

In addition to progressing through the larval stages, under unfavourable conditions, such as high temperature, low food availability, high population density and/or pheromones, worms can take an alternative developmental path at the L2 molt resulting in an extended arrested stage, known as *dauer* larva diapause [19] [20]. The *dauer* larvae can survive almost 4-8 times longer than the normal ones [21], approximately 70-130 days, and can resume normal development when favourable conditions are achieved (e.g., food availability). The *dauers* are generally thinner and are darker as compared to the developing worms. They have sealed buccal openings, reduced pharyngeal pumping and arrested germline development.

The IIS pathway plays a major role in controlling the *dauer* formation [22]. Mutations of the IIS components; *daf-2, age-1, pdk-1,* or both *akt-1; akt-2* can result in constitutive *dauer* phenotype. Induction of the transcription factor DAF-16 plays a major role in this phenotype [23] [24]. The other major parallel pathway to control *dauer* arrest is the TGF-β signalling pathway, which includes the TGF- β -like ligand *daf-7,* and *daf-1*, *daf-3* as well as *daf-5* [22].

1.4. The discovery of long-lived IIS mutants

The discovery of the long-lived mutant *age-1* [25] and that the *daf-2* mutants live twice as long initiated a dazzling cascade of discoveries [4] and stimulated rapid growth of the field of lifespan genetics. Moreover, the same *daf-2* mutant that show extended lifespan had already been known to cause a constitutive *dauer* formation (Daf-c phenotype) at restrictive temperatures. The presence of *daf-16* gene is essential for the Daf-c phenotype of the *daf-2* alleles [26] and for the longevity phenotype of the mutants [4]. Today, the fact that the lifespan extension relies completely on a single transcription factor has

provided researchers the expectation that the cause of aging can be found by studying long-lived IIS mutants and that this finding can be within reach.

1.5. DAF-16/FoxO Transcription Factor in aging and longevity

C. elegans insulin/insulin-like growth factor/IGF-1 signaling (IIS) is one of the most known aging-regulatory pathways whose components are studied extensively. IIS's function as a lifespan regulatory pathway is evolutionarily conserved in flies, mice, and most likely in humans [27] [28].

The Forkhead O transcription factors (FoxO) is a crucial downstream regulator that plays an essential role in aging and longevity of metazoans [29] [30]. They are the central regulators of growth, metabolism, stress, DNA damage repair, apoptosis, cell-cycle control in many organisms [31]. DAF-16 is the sole FOXO homolog in *C. elegans*, has multiple isoforms (a, b, and d/f) and a high degree of homology to FOXO3A, a member of the FOXO family [32] [33].

Remarkably, the molecular studies revealed that *age-1* and *daf-2* encode a phosphoinositide-3 kinase (PI3K) and an insulin/IGF-1 receptor, respectively: these are the key upstream components of IIS that culminate in activation of AKT-1, which phosphorylates DAF-16 and blocks its nuclear entry resulting in sequestration in the cytosol [34] (Figure 1).

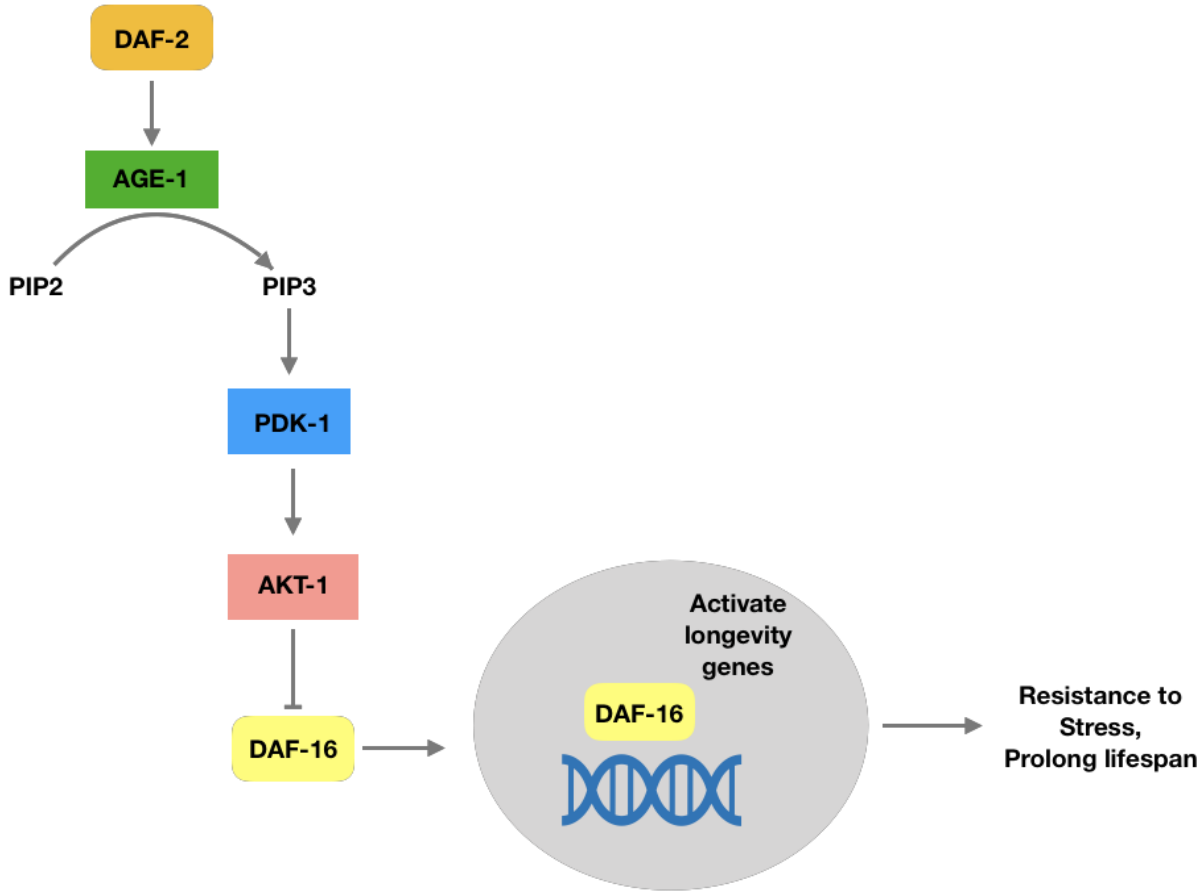


Figure 1. *C. elegans* IIS pathway

Inhibition of IIS is known to promote longevity in *C. elegans*: DAF-2's reduced function results in downstream kinase cascade inactivation, starting with AGE-1/PI3K [35]. Down-regulation of AGE-1 contributes to 3-phosphoinositide-dependent kinase 1 (PDK-1) inactivation, possibly due to a decrease in the PI(3, 4, 5)P3/PI(4, 5)P2 ratio [36] [37]. The Akt / protein kinase B (PKB) family members AKT-1 and AKT-2, are, in turn, down-regulated [36] [38].

Therefore, reduction of function or mutation or RNAi of the upstream components of the IIS, such as *daf-2*/IGFR, *age-1*/PI3K, *pdk-1*/PDK-1 or *akt-1/2*/AKT-1/2, reduce DAF-16/FoxO phosphorylation, resulting in its nuclear translocation and subsequent transcriptional activation of DAF- 16/FoxO target genes [39]. In turn, the worms are more stress-resistant and have an increased life span [39].

The IIS pathway regulates lifespan across species, from invertebrates to mammals: more extensively studied in *C. elegans*, *Drosophila melanogaster* (fruit fly) [40] [41] and *Mus musculus* (mice) [42] [43] . In mammals, the IGF-1 is produced in the liver and regulated by the growth hormone (GH). Reduction in the GH signaling or loss of function of the GH receptor renders the mice long-lived. For instance, in Snell (PIT-1 mutants) and Ames mice (PROP-1 mutants), lifespan can increase approximately to 50% in males and 64% in females relative to their wild- type controls [44] [45]. Other examples include, lit/lit mice (GH-releasing hormone receptor mutants) [46]. Remarkably, klotho gene appears to induce insulin-sensitivity and its over-expression is shown to repress the IIS and enhance longevity [47] [48].

The role of IIS in controlling lifespan may also extend to humans. An exploratory study on the Ashkenazi Jewish centenarians identified no changes in the IGF gene, but polymorphisms in *Igf1r* that are associated with longevity [49]. Moreover, polymorphisms in FOXO3A have been shown to be associated with longevity in humans [50].

The other two conserved evolutionary regulators that have been involved in regulating lifespan are the mechanistic target of rapamycin (mTOR) kinase and Sirtuins. TOR associates in two different complexes: TORC1 and TORC2. TORC1-mediated longevity is dependent on DAF-16/FoxO [39]. The IIS, TOR and Sirtuins are inter-connected to each other through AMPK, which is an energy-sensing molecule that responds to stimuli of decreased energy availability as well as reduced glucose levels. AMPK is triggered in starving conditions, which affects intracellular metabolism, leading to an increase in NAD^+ levels, with an increase in SIRT-1 activity [51]. Once the sirtuins are activated, they regulate FOXO transcriptional activity, thereby linking SIRT-1 to the IIS pathway. In converse, DAF-16 is important for AMPK to function in oxidative stress resistance and longevity [52] [53] . AMPK negatively regulates mTORC1 through phosphorylation and activation of TSC2 (tuberous sclerosis complex 2) [54].

DAF-16/FOXO is therefore involved in integrating signals from these pathways to regulate aging and longevity by shuttling from cytosol to the nucleus.

1.6. Preliminary data (the screen)

In a previous RNAi screen (a candidate RNAi screen targeting genes related to RNA metabolism) set to identify new regulators of DAF-16 expression, Evandro Araújo de Souza, under the guidance of Marcelo A. Mori (UNICAMP) and Adam Antebi (Max Planck Institute for Biology of Aging), found some genes that control GFP::DAF-16 nuclear localization in a worm that lacks the germline (*glp-1* mutant). Those genes were *adr-1* (3 out of 3 replicates), *nrde-1* (3 out of 3 replicates), *wago-1* (3 out of 3 replicates), *pgl-1* (2 out of 3 replicates), *mes-4* (3 out of 3 replicates), *mpk- 1* (2 out of 3 replicates), *set-25* (2 out of 3 replicates), *hda-1* (1 out of 3 replicates), *hda-4* (1 out of 3 replicates), *ire-1* (1 out of 3 replicates), *mut- 7* (1 out of 3 replicates), and *unc-130* (1 out of 3 replicates). In summary, worms that lack the germline and express a transgene were DAF-16 is fused to GFP at the N-terminal were exposed to RNAi and nuclear vs. cytoplasmic GFP localization was scored. In control worms, *i.e.* treated with empty vector, the absence of germline resulted in 100% of intestinal cells in which DAF-16 was in the nucleus. When RNAi targeting the genes above was used, at least in one out of three replicates, DAF-16 was shuttled to the cytoplasm.

In order to validate whether these genes might participate in the regulation of DAF-16 function, they tested whether RNAi against some of these genes can also downregulate a DAF-16 target gene (i.e., sod-3). As shown in Table 1, *adr-1*, *hda*-1, *nrde-1*, *set-25* and *wago-1* RNAi reduced at some level the expression of a *sod-3* reporter construct (sod-3p::GFP). Furthermore, they tested whether the same genes can regulate SKN-1 nuclear localization. SKN-1 is yet another stress response transcription factor that is required for the germline longevity phenotype [55] [56] [57]. Interestingly, *adr-1, hda-1, set-25* and *wago-1* RNAi also -reduced SKN- 1 nuclear translocation, while *nrde-1* seemed to be specific for DAF-16 regulation.

Condition	***sod-3p*::GFP**	**SKN-1::GFP**
OP50	High	Nuclear
L4440	High	Nuclear
ADR-1i	**Low**	**Cytoplasmic**
DAF-16i	**Low**	n.d.
HDA-1i	**Low**	**Cytoplasmic**

MES-4i	High	Nuclear
NRDE-1i	**Low**	Nuclear
PGL-1i	High	Nuclear
SET-25i	**Intermediate**	**Intermediate**

Table 1. Validation of genes necessary for DAF-16 activity in *glp-1* mutant. All clones were tested at least three times. n.d. Not done.

Of all the genes picked up by the screen, here we decided to focus on *adr-1, nrde- 1, wago-1,* and *set-25.* The inclusion criteria were mainly because their ability to regulate DAF-16 has been confirmed by multiple experiments and reagents were available.

ADR-1 and ADR-2 are the two isoforms of adenosine deaminases (ADARs) in *C. elegans*. ADARs, commonly known as RNA-editing enzymes, deaminate adenosines to create inosines in double-stranded RNA. *adr-1* is mostly expressed in the nervous system (e.g., sensory neurons and cilia, the ventral nerve cord, motor neurons, and interneurons), the embryos, and the developing vulva of the *C. elegans*. It is required for normal chemotaxis, vulval development and to prevent the silencing of transgenes in somatic tissues by RNAi. ADAR loss of function mutations in *C. elegans* render worms short-lived, which is consistent with ADR-1 positively regulating DAF-16 [58] [59] [60] [61].

nrde-1 encodes a novel protein conserved amongst nematodes. NRDE-1 has dual associations in the nuclear RNAi pathway: one is to act in association with the other Nrde components on nascent pre-mRNA transcripts at the site of RNA Pol II activity and the other at a downstream level at the chromatin acting closer to the site of H3K9me3 silencing [62]. NRDE-3 is involved in the recruitment of NRDE-1 to chromatin and therefore for transcriptional inhibition and H3K9 trimethylation [63]. There appears to be two distinct forms of inherited RNAi: inheritance of somatic RNAi for one or a few generations and inheritance of germline RNAi, over multiple generations [64]. The WAGO Argonaute NRDE- 3 (Nuclear RNAi defective) pathway is the mediator of transcriptional gene silencing in the soma. NRDE-3, which contains a nuclear localization signal (NLS) is triggered by WAGO 22G RNA binding to enter the

nucleus and to bind its targets. Loss of *nrde-3* leads to accumulation of pre-mRNA and mRNA levels of its targets [65]. NRDE-3 is required for the inheritance of somatic silencing triggered by exo-RNAi for a single generation and in the progeny NRDE-3 is responsible for the accumulation of secondary WAGO 22G RNAs to re-establish H3K9me3 marks [66].

NRDE-2 is a nuclear-localized, evolutionarily conserved protein. It functions in association with NRDE-3 for the transport of siRNAs from cytosol to nucleus. It is recruited by NRDE-3/siRNA complexes to the RNAi targeted nascent transcripts within the nucleus [67]. NRDE-4 is a conserved, nematode-specific protein which plays an important role in the Nuclear RNAi pathway.

WAGO-1 is an ortholog of members of the human Argonaute/PIWI family and is localized to the P granule and expressed in the germline [68]. WAGO-1 is involved in the endogenous small interfering RNA (endo-siRNA) pathway and interacts with secondary 22G-RNAs. In the germline, it functions in a genome surveillance system and silence transposons, pseudogenes, aberrant transcripts and regulate gene expression [69].

SET-25 encodes a putative histone H3 lysine-9 methyltransferase, with a C- terminal SET domain but no apparent non-nematode orthologs. SET-25 is required both for normal axonal guidance during development and for a normally low somatic mutation rate [70]. It was observed that the deletion of SET-18 (yet another histone methyltransferase) resulted in increased lifespan as well as oxidative stress resistance in a DAF-16 dependent manner [71].

2. OBJECTIVE

The main aims of this project are to investigate the interaction of NRDE-1, WAGO-1, ADR-1 and SET-25 with the IGF-1/Insulin Signalling Pathway (IIS); their possible involvement in DAF-16 translocation, *dauer* formation and lifespan.

3. MATERIALS AND METHODS

3.1. General methods and *C. elegans* stock maintenance

For the experiments, *C. elegans* were used. They were obtained from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota. All lineages were already available in the laboratory of Prof. Marcelo A. Mori. The *C. elegans* nematode strains used in the experiments were maintained in incubators at 15- 20°C in Petri dishes with semisolid Nematode Growth Media (NGM) medium containing *E. coli* OP50-1 bacterial layer [except in the experiments involving RNAi in which the HT115 (DE3) bacteria were used]. OP50 plates were supplemented with 100 μg/mL streptomycin, whereas, the RNAi plates with 1mM IPTG, 100 μg/mL tetracycline and 100 μg/mL ampicillin. The stocks were maintained by periodically transferring the worms to a fresh plate by chunking a section of the agar. A thin platinum wire was used for transferring/picking the worms.

3.1.1. Strains

N2 (wild-type), CB1370 *daf-2 (e1370) III*, MAM71 *muIs109 (daf- 16p::GFP::DAF-16 cDNA + odr-1p::RFP)*, CF2570 *[daf-16(mu86) I; daf- 2(e1370) III; muIs142 (ges- 1p::GFP::daf-16(cDNA) + odr-1p::RFP)]*, TJ356 *zIs356 [daf-16p::daf- 16a/b::GFP + rol-6(su1006)] IV*, CF1407 *[daf-16(mu86) I; muIs71 ((pKL99) daf-16ap::GFP::daf-16a(bKO)) + rol-6(su1006)]*, GR1310 *akt-1(mg144) V*, GR1318 *pdk-1(mg142) X*, TJ1052 *age-1(hx546) II*, CF1553 *muIs84 [(pAD76) sod-3p::GFP + rol-6(su1006)]*.

3.2. Synchronization

C. elegans were synchronized by the bleaching method at the L1 stage. Worms were allowed to grow until adult stage. Gravid adults were transferred to 15 ml tube by washing plates with M9 buffer and were centrifuged for 2 minutes at 400xg (~1500 rpm on a standard table centrifuge) at room-temperature. The pellet was obtained and supernatant was discarded. The pellet was washed 1-3 times until the buffer appeared clear and free of bacteria. The bleaching solution (0.5 mL of 5 M NaOH and 1 mL of 5% of sodium hypochlorite solution, completing

the volume with sterile water to 5mL) was added and agitated for 3-10 minutes, until the adult tissues were ruptured and eggs were released. M9 buffer was added to the tube and a quick centrifuge was done for 1 minute at 400 x g and discard supernatant. The pellet was washed three more times by filling the tube with M9 buffer. 1-5 ml of M9 buffer was added to the pellet and incubated at 20°C for ~20-24 hours for L1 offspring hatching, which were later transferred to the NGM plates with OP5O/HT115.

3.3. RNA interference

The RNAi by feeding technique was made according to Kamath R.S. et al. [72] and the clone used for *adr-1, nrde-1, wago-1, set-25* silencing were obtained from Volovik Y et al [73] and *rict-1, akt-1,nrde-2, nrde-3 and nrde-4* from Vidal's library. For the silencing of *adr-1, nrde-1, wago-1* and *set-25* genes, worms were synchronised and the eggs obtained were be plated on RNAi [genes] or RNAi Control [luciferase] plates. For all the experiments worms were kept in RNAi from L1 stage.

3.4. Lifespan assay

For the measurement of the lifespan, about 80-100 synchronized worms were monitored daily, from day 0 (zero) of adulthood, using a light stereoscope, according to Ferraz RC et al [74]. The experiments were performed at 20 -25°C, the physiological temperature for the worm. We will use 100 μg/mL of streptomycin and 0.25 μg/mL of amphotericin B to prevent contamination and 0.5 μg/mL 5-fluoro-2-deoxyuridine-FUdR (Sigma), an inhibitor of mitosis, to prevent offspring development and overpopulation of the plaques.

3.5. Gene Expression

The gene expression was performed according to the protocol of Bratic I. et al [75]. In summary, pools of 100 3-day adult worms were transferred to 1.5 mltubes and the RNA extraction was performed following the Trizol® Reagent (Invitrogen) manufacturer's instructions. Briefly, after the trituration of the sample in the presence of Trizol, 1/5 (v / v) chloroform was added to the tube. After 10 minutes standing at room temperature the samples were centrifuged in a refrigerated Eppendorf centrifuge at 12,000 x g for 15 min at 4°C. After centrifugation, the colorless phase of the tube were transferred to a new tube for the

precipitation of RNA in the presence of isopropyl alcohol. After another centrifugation at 12,000 x g, 10 min, 4°C, a precipitate containing RNA could be identified. Ethanol 75% was added to wash the precipitate and then the ethanol removed and sterile water was added. The RNA was quantified in NanoDrop 2000c spectrophotometer. To perform the reverse transcription, we used 0.2-1µg of total RNA. The reaction was be conducted using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. After the synthesis of the cDNAs, we quantified the expression of the *daf-16a, daf-16b, scl-1, mdl-1, sod-3, age-1, akt-1, pdk-1, daf-18* by real-time PCR. The reaction was conducted using the Maxima SYBR Green Master Mix (Fermentas), primers at the final concentration of 250 nM, and fluorescence was detected using the Applied Biosystems 7500 Real-Time PCR System. The amplification protocol used was: 50 ° C for 2 min, 95 ° C for 10 min and 40 cycles of 95 ° C for 15 s, 60 ° C for 20 s and 72 ° C for 30 s. *his-10* was used as endogenous control.

3.5.1. Primer sequences

DAF-16a _F :CACCACCATCATACCACGAG

DAF-16a _R :TGCTGTGCAGCTACAATTCC

DAF-16b _F :AGACAACGACCAGACGGAAC

DAF-16b _R :GGATCGAGTTCTTCCATCCA

SCL-1 _F :CAATCAAGCATTGTGGATGC

SCL-1_ R :CGCAGAAGTCCAAGACCAAT

MDL-1 _F :AACTTCAAGCCGACGAAGAA

MDL-1_ R :ATTGGACCCCTTGGGATAAG

SOD-3 _F :TCGGTTCCCTGGATAACTTG

SOD-3_R :CATAGTCTGGGCGGACATTT

AGE-1 _F :GACGGAACTCCCGACGTATC

PDK-1_F :TGATGTTCGCAAGCGGTACT

PDK-1_R :TTGCTGGAAACTCAGTGAGGG

AKT-1_F :CCCAGCACGTCCTTCTCCTA

AKT-1_R :CTCCACCA TTCGCAAACTGC

DAF-18_F :GTCGTGGGCTCGGATAGAAG

DAF-18_R :TCTGTTTTGCAGGCTAAGGA

3.6. GFP fluorescence and microscopy (*GFP reporter analyses)*

The experiments were performed in a similar way according to Ferraz et al [74]. The synchronized worms were maintained in OP50-1 or RNAi plates until adulthood. 10-12 worms of the appropriate age were picked into the wells of microtitre plates (flat bottom Greiner). 80 μL of M9 media (22 mM Na_2HPO_4, 22 mM KH_2PO_4, 85 mM NaCl, 1 mM $MgSO_4$) was added in the wells and the worms were immobilized using 0.1% sodium azide and images were acquired using the Cytation V microscope (Biotek). A constant gain was set in the plate reader for each condition. Images were analysed using ImageJ and integrated density was quantified. For the nuclear localization assays, the frequency of nucleated DAF-16::GFP in worms expressing the *pdaf-16::daf-16::gfp* transgene were qualitatively graded as high, medium or low levels based on the number of cells with nucleated versus cytoplasmic DAF-16::GFP [76].

3.1. *Dauer* Assay

Dauer assays was performed in *daf-2* mutants. These mutants spontaneously enter in *dauer* when kept at 25°C. Synchronized eggs using the bleaching method were placed onto NGM plates and grown at 25°C. Worms were scored for the presence of *dauer* and non-*dauer* under the microscope after 60-65 hours.

3.1. Statistical Analysis

C. elegans survival under various conditions and of various genotypes was monitored as described in the figures. For the statistical analysis of life span we used Log-rank test (Mantel-Cox). For the other experiments we used the Two- tailed student *t* test, for comparisons between 2 groups, and the Two-way ANOVA test and Bonferroni's Post-test, for comparisons between more than 2 groups. The differences were considered statistically significant when the P value is < 0.005. The software used was GraphPad Prism (Version 8).

4. RESULTS AND DISCUSSION

4.1. Effects of *nrde-1, wago-1, adr-1* and *set-25* knockdown on:

4.1.1. DAF-16 translocation

A key step of DAF-16 regulation is the translocation of DAF-16 from the cytoplasm to the nucleus. First, aiming to validate whether the expression of DAF-16 is dependent on the function of the candidate genes, we silenced them using the RNAi feeding mechanism in *daf-2* mutant worms expressing the DAF- 16::GFP construct and wild type worms expressing the same transgene. We therefore monitored the DAF-16::GFP localization in L4 worms. To further induce DAF-16::GFP nuclear translocation, we exposed the worms to heat shock treatment at 34°C for 1.5 hours. As the DAF-16 function is dependent on its presence in the nucleus of the cells, trying to assess this, we measured the presence of the DAF-16 in the nucleus of the worm, scoring by GFP fluorescence. We performed the heat shock experiment with a few different DAF- 16 reporter transgenes to avoid any transgene bias (Figure 2- 5). Here we used RNAi against Luciferase as the control. The observation was that ADR-1, WAGO-1, SET-25 and especially NRDE-1 inhibited GFP::DAF-16 nuclear localization, so it can be expected that these proteins may somehow be interacting with the IIS to regulate DAF-16 function and longevity.

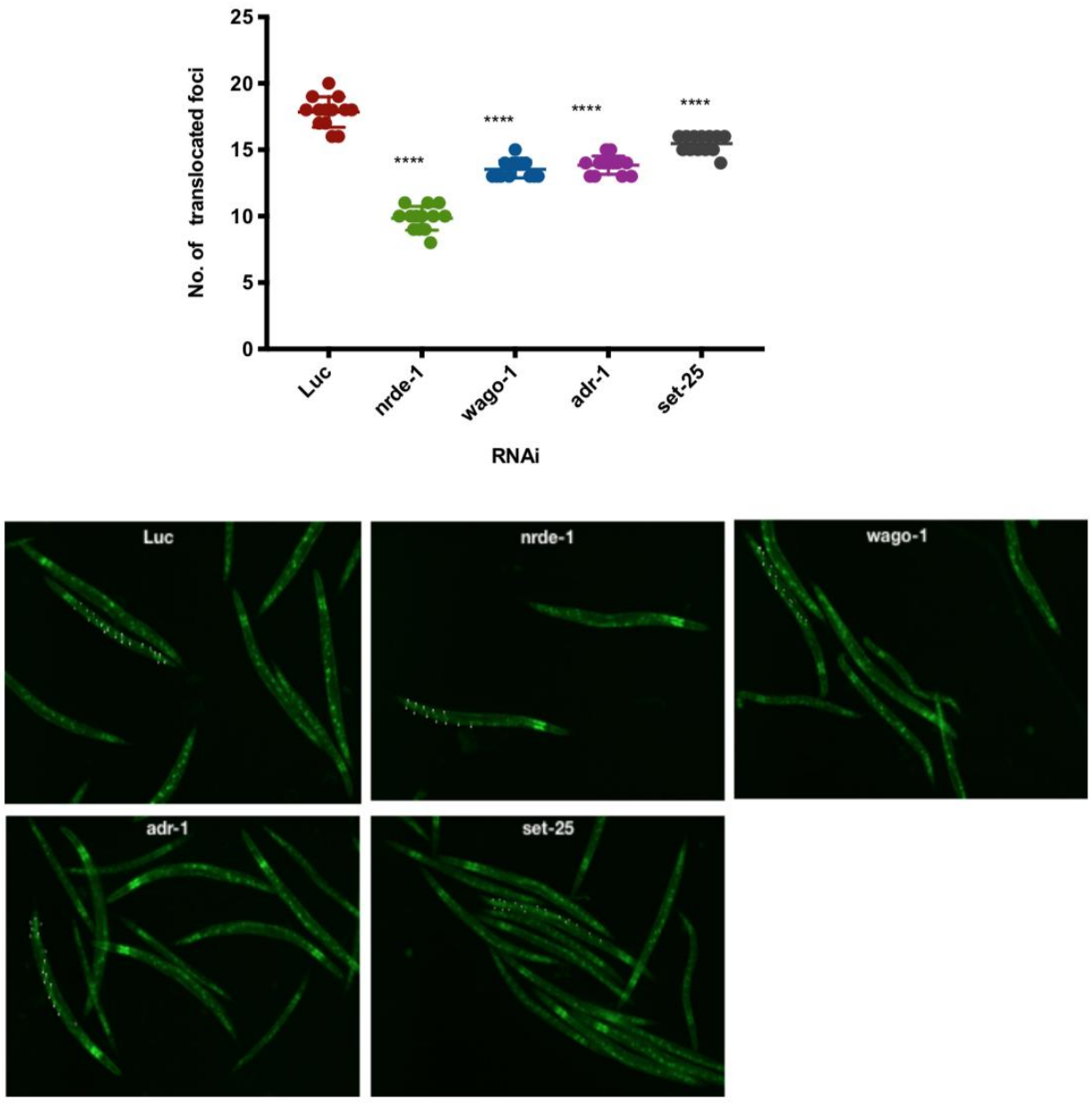


Figure 2. DAF-16 translocation assay in heat stressed MAM71 (DAF-16::GFP) worms. The number of DAF-16::GFP positive nucleus (the translocation of DAF-16 from the cytoplasm to the nucleus) was scored using Cytation V. *nrde-1, wago-1, adr-1, set-25* genes were silenced and luciferase was used as a control. ****$p < 0.0001$. Two-tailed student *t*-test was used for comparison with the control (*luciferase*). Representative images of DAF-16 translocation are below each graph. MAM71 worms were maintained at 20°C and heat-shocked at 34°C for 1.5 hours on the first day of adulthood), N=13.

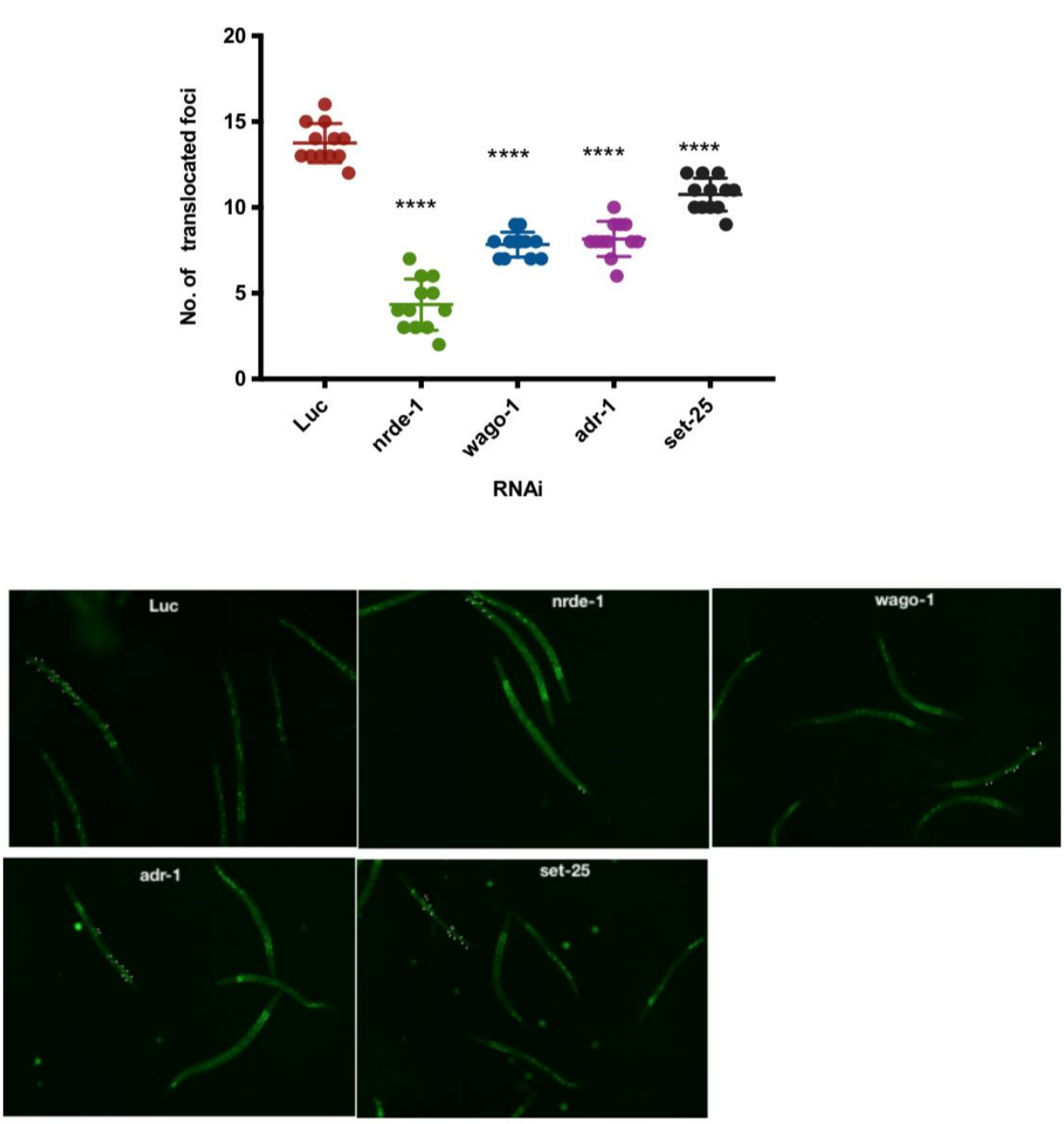


Figure 3. DAF-16 translocation assay in CF2570 *[daf-16(mu86) I; daf-2(e1370) III; muIs142 (ges-1p::GFP::daf-16(cDNA) + odr-1p::RFP)]* worms. The number of DAF-16::GFP positive nucleus (the translocation of DAF-16 from the cytoplasm to the nucleus) was scored using Cytation V. *nrde-1, wago-1, adr-1, set-25* genes were silenced and luciferase was used as a control. ****$p < 0.0001$. Two-tailed student *t*-test was used for comparison with the control (*luciferase*). Representative images of DAF-16 translocation are below each graph. CF2570 worms were maintained at 15°C till L2 and then transferred to 25°C, N=12.

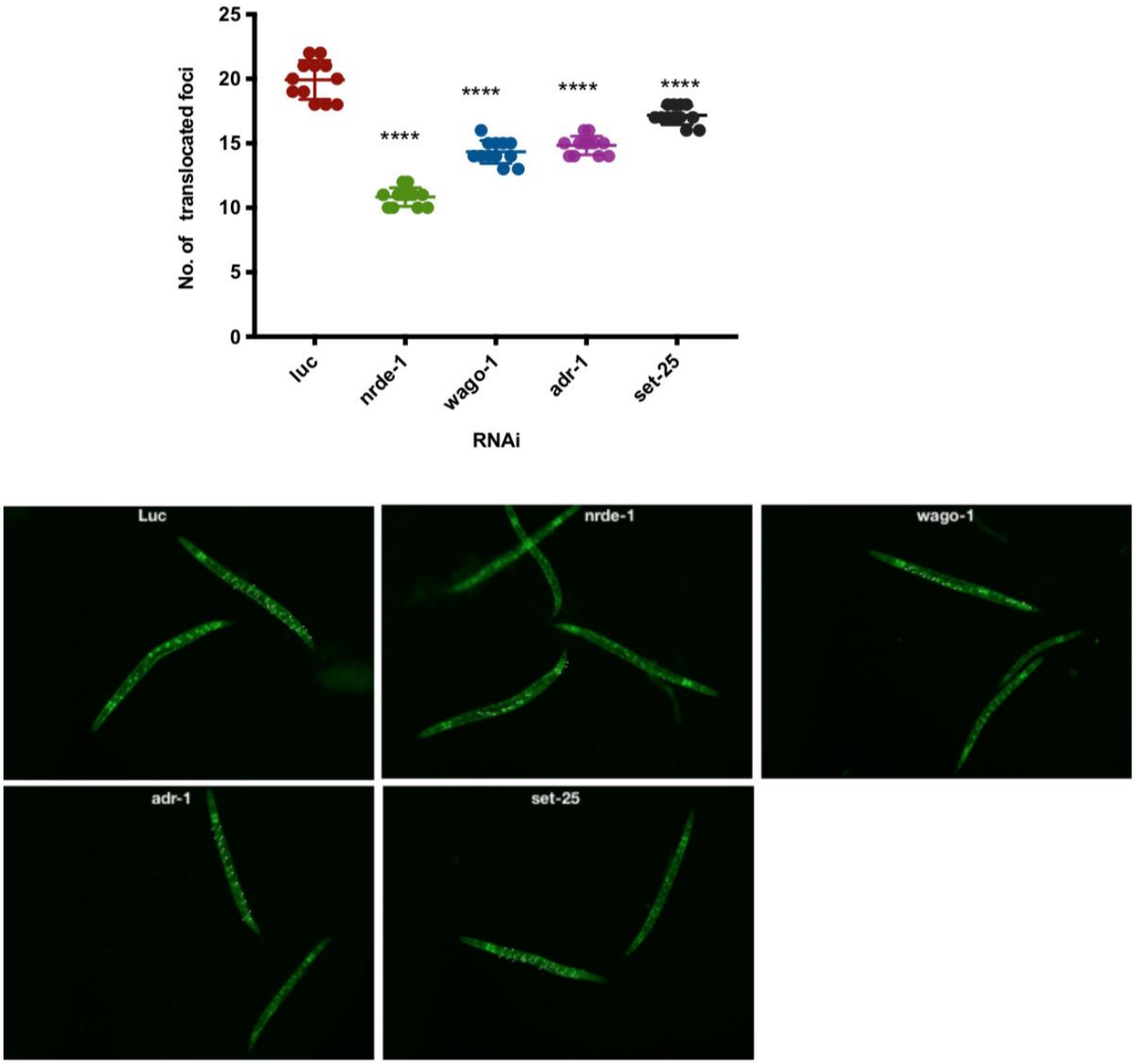


Figure 4. DAF-16 translocation assay in heat stressed TJ356 *zIs356 [daf-16p::daf-16a/b::GFP + rol-6(su1006)] IV* worms. The number of DAF-16::GFP positive nucleus (the translocation of DAF-16 from the cytoplasm to the nucleus) was scored using Cytation V. *nrde-1, wago-1, adr-1, set-25* genes were silenced and luciferase was used as a control. ****$p < 0.0001$. Two-tailed student *t*-test was used for comparison with the control (*luciferase*). Representative images of DAF-16 translocation are below each graph. TJ356 worms were maintained at 20°C heat- shocked at 34°C for 1.5 hours on the first day of adulthood, N=12.

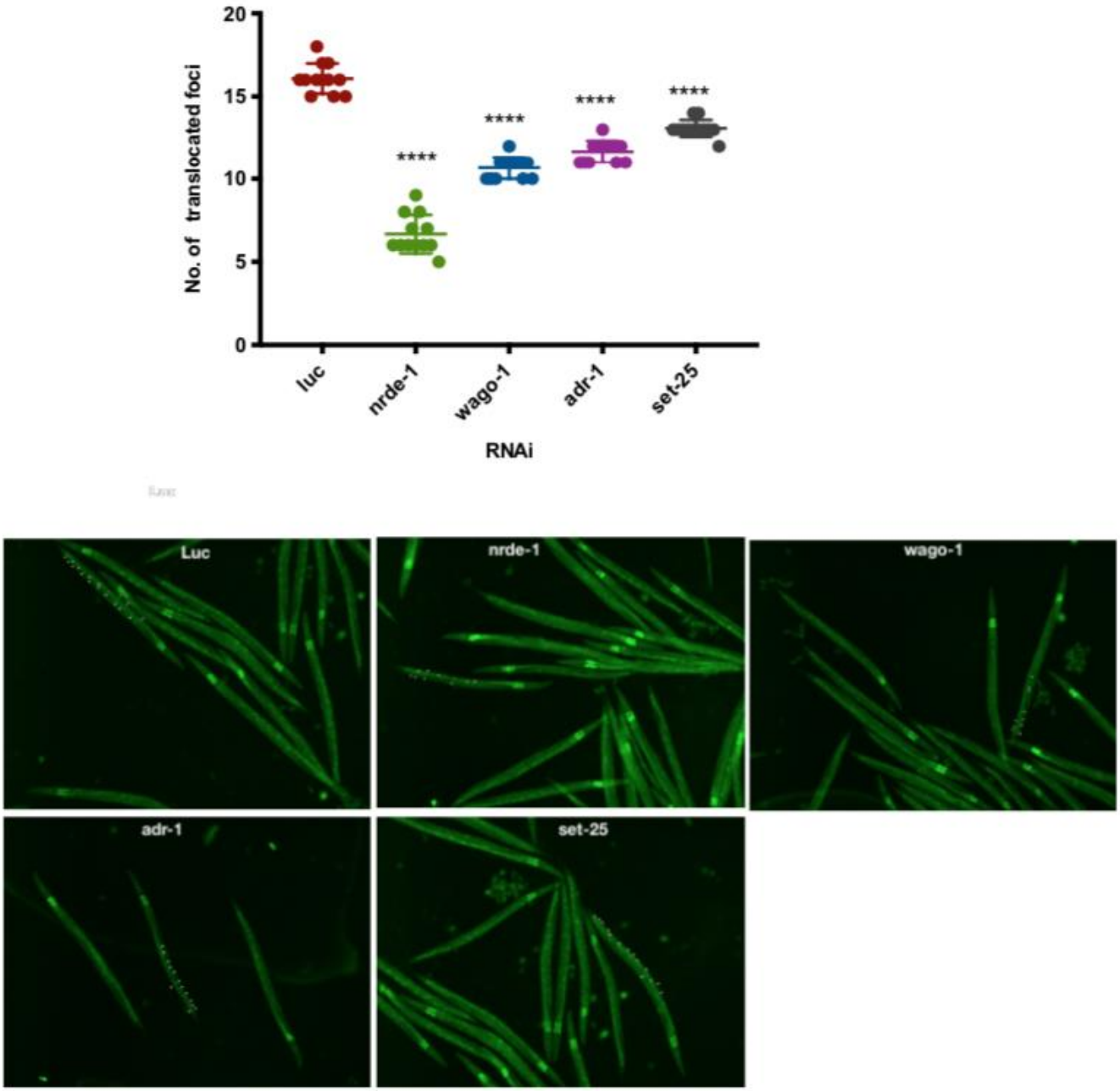


Figure 5. DAF-16 translocation assay in heat stressed CF1407 [*daf-16(mu86) I; muIs71 ((pKL99) daf-16ap::GFP::daf-16a(bKO))* + *rol- 6(su1006)*] worms. The number of DAF-16::GFP positive nucleus (the translocation of DAF-16 from the cytoplasm to the nucleus) was scored using Cytation V. *nrde-1, wago-1, adr-1, set-25* genes were silenced and luciferase was used as a control. ****$p < 0.0001$. Two-tailed student *t*-test was used for comparison with the control (*luciferase*). Representative images of DAF-16 translocation are below each graph. CF1407 worms were maintained at 20°C and heat-shocked at 34°C for 1.5 hours on the first day of adulthood, N=12.

We then sought to measure the 'integrated density' of GFP as a measurement of the level of expression of the transgene. We could see a consistent decrease in DAF-16 level (i.e. GFP) in these worms upon silencing with *nrde-1* RNAi but not with the others (Figure 6). However, this decrease was not as great as the decrease in DAF-16 translocation, suggesting that at least in part DAF-16 is controlled at the post-transcriptional level.

A similar experiment was conducted using the sod-3::GFP construct (SOD-3 is a target of DAF-16). Consistent with an inhibition of DAF-16 translocation, we noticed a decrease in the levels of GFP in this strain when the candidate genes were silenced, particularly NRDE-1 (Figure 7).

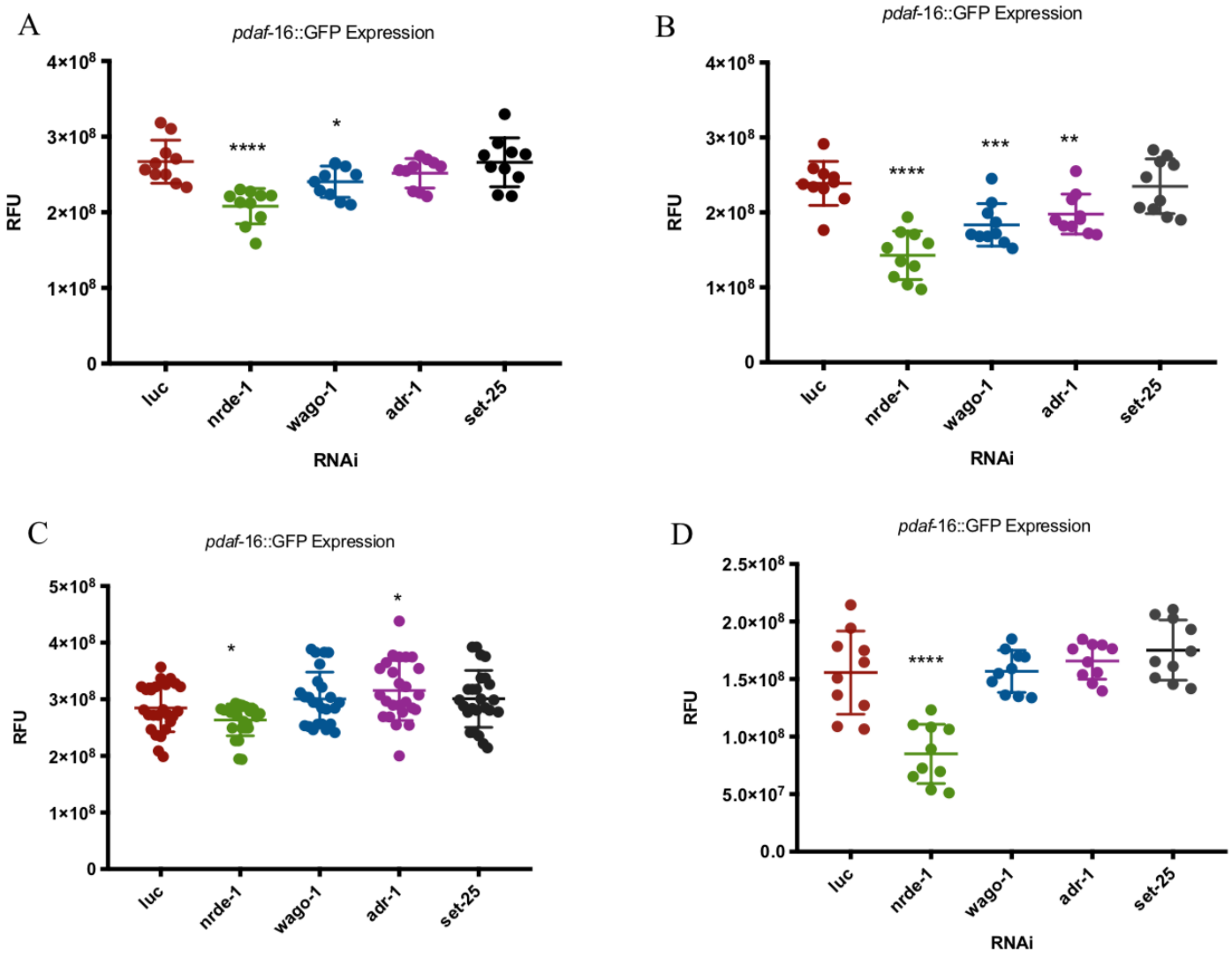


Figure 6 Daf-16::GFP expression quantification using Image J. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Two-tailed student t-test used for comparison with the control.

A. MAM71 DAF-16::GFP (maintained at 20°C and heat-shocked at 34°C for 1.5 hours on the first day of adulthood), N=25

B.CF2570*[daf-16(mu86)I;daf-2(e1370)III;muIs142(ges-1p::GFP::daf-16(cDNA)+ odr1p::RFP)*] (maintained at 15°C till L2 and then transferred to 25°C), N=10

C. TJ356*zIs356[daf-16p::daf-16a/b::GFP+rol-6(su1006)]IV*(maintainedat20°Cheat- shocked at 34°C for 1.5 hours on the first day of adulthood), N=10

D. CF1407 [*daf-16(mu86) I; muIs71 ((pKL99) daf-16ap::GFP::daf-16a(bKO)) + rol-6(su1006)*] (maintained at 20°C and heat-shocked at 34°C for 1.5 hours on the first day of adulthood), N=12

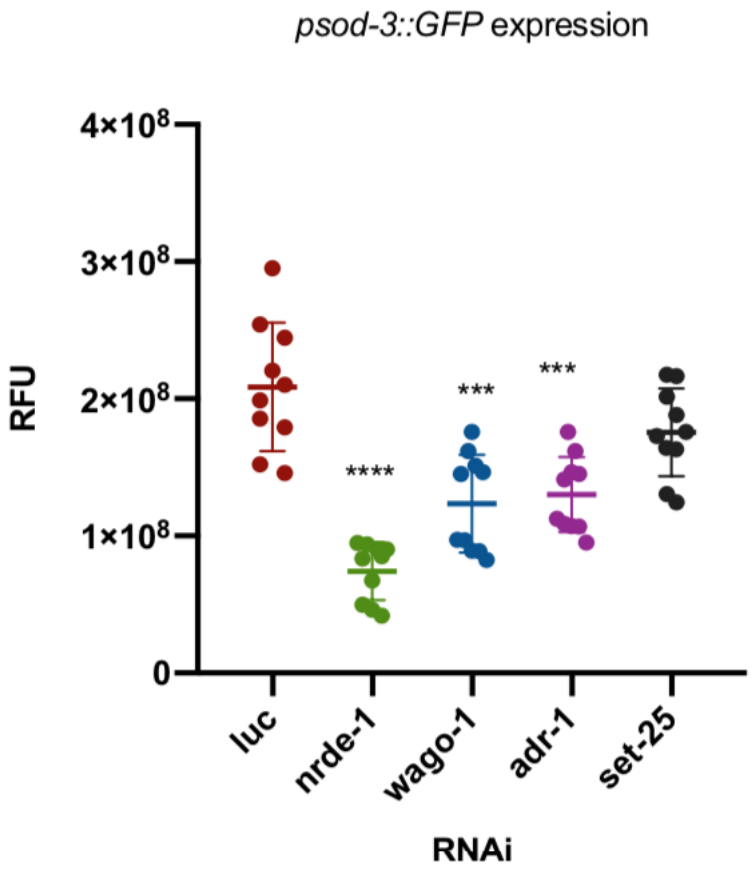


Figure 7. *sod-3*::GFP expression quantification using Image J. *nrde-1, wago-1, adr-1, set-25* genes were silenced and luciferase was used as a control. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Two-tailed student *t*-test was used for comparison with the control.

CF1553 *muIs84 [(pAD76) sod-3p::GFP + rol-6(su1006)]* (maintained at 20°C and heat-shocked at 34°C for 1.5 hours on the first day of adulthood), N=10

4.1.2. *Dauer* formation

Another way to test DAF-16 function is by measuring the *dauer* entry. *Dauer* is a resistant stage on the nematode development and it can be promoted by mutations like in *daf-2*. Importantly, *dauer* entrance in these mutants is entirely dependent on DAF-16 [26] and the *daf- 2(e1370)* mutants are thermo-sensitive, i.e. they develop into *dauers* at higher temperatures (25°C) but not at lower temperatures (15°C) [77]. To test the importance of *nrde-1, wago-1, adr-1* and *set-25* on the *dauer* formation, we silenced these genes in *daf-2* mutants and evaluated the presence of the *dauer* stage in the population. Silencing of the candidate genes resulted in a significant decrease of *dauer* population (Figure 8). Again, the effect was

much stronger in case of NRDE-1. Therefore, this result suggests that knocking-down the candidate genes suppresses the *dauer* arrest phenotype of *daf-2*, which suggests that the genes are major regulators of endogenous DAF-16.

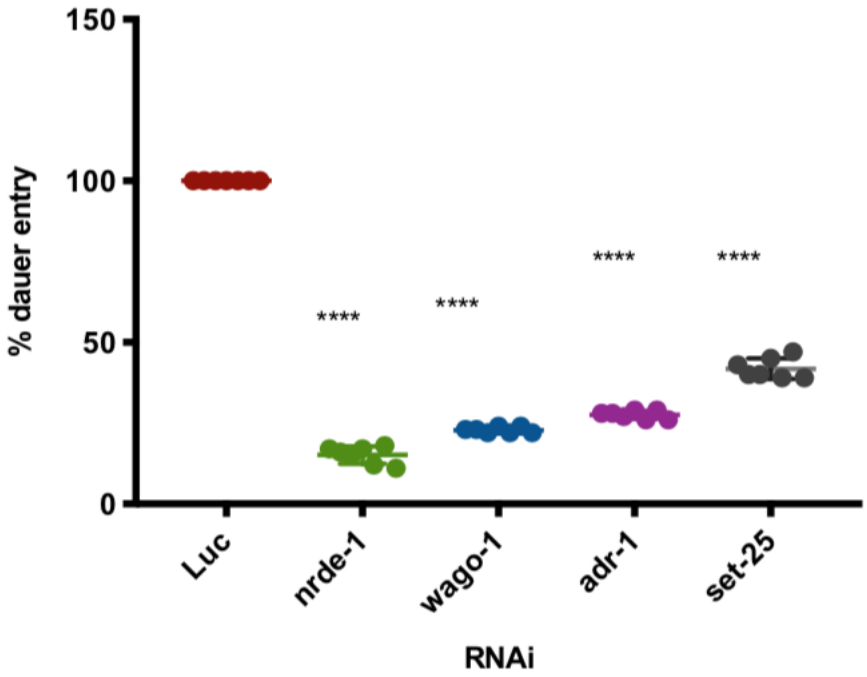


Figure 8. Dauer assay of *daf-2(e1370)* mutants. *nrde-1, wago-1, adr-1, set-25* genes were silenced and luciferase was used as a control. N=100 and number of replicates= 7. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Two-tailed student *t*-test was used for comparison with the control.

4.1.3. Endogenous transcription activity of DAF-16

Among DAF-16/FoxO upregulated target genes, some of them code for proteins required to deal with various forms of stress. Superoxide dismutase (*sod-3*), for instance, fights oxidative stress [78], while *scl-1* is required for lifespan extension in *daf-2* and *age-1* mutants [79]. Also, *scl-1* expression is dependent on DAF-16, and is the first example of a putative secretory protein that positively regulates longevity and stress resistance [80]. The isoforms of DAF-16 *daf-16b* functions in *dauer* formation, while *daf-16a* and *daf-16d/f* functions in lifespan regulation [81]. DAF-16 also directly activates transcription of *mdl-1*, which is an ortholog of human MXD3 (MAX dimerization protein 3) and MXI1 (MAX interactor 1, dimerization protein); exhibits protein heterodimerization activity; contributes to sequence-specific DNA binding activity; is involved in determination of adult lifespan and regulation of cell differentiation; localizes to the nucleus and transcription factor complex; is expressed in the alimentary system, the muscular system, and the nervous system; is used to study Parkinson's disease; and the human ortholog(s) of this gene are implicated in prostate cancer [82]. However, another study suggests that DAF-16 downregulates *mdl-1* mRNA levels, indicating a more complex or condition dependent relationship between the IIS pathway and *mdl-1* [83].

To assess endogenous transcription activity of DAF-16 and confirm its interaction with the above mentioned genes, we used RT-qPCR to measure the DAF-16 target genes *sod-3, mdl-1* and *scl-1* ¾ along with the isoforms *daf-16a* and *daf-16b* ¾ in wild type worms (N2) subjected to either control RNAi or RNAi against the candidate genes *nrde-1, wago-1, adr-1* and *set-25*. These genes were silenced by RNAi feeding mechanism and worms were subjected to heat- shock at 34°C for 1.5 hours on the first day of adulthood before the RNA extraction. Consistent with the hypothesis that endogenous DAF-16 is regulated by the candidate genes,

we could see a down-regulated expression of the DAF- 16 target genes, which was much more pronounced when NRDE-1 was silenced (Figure 9).

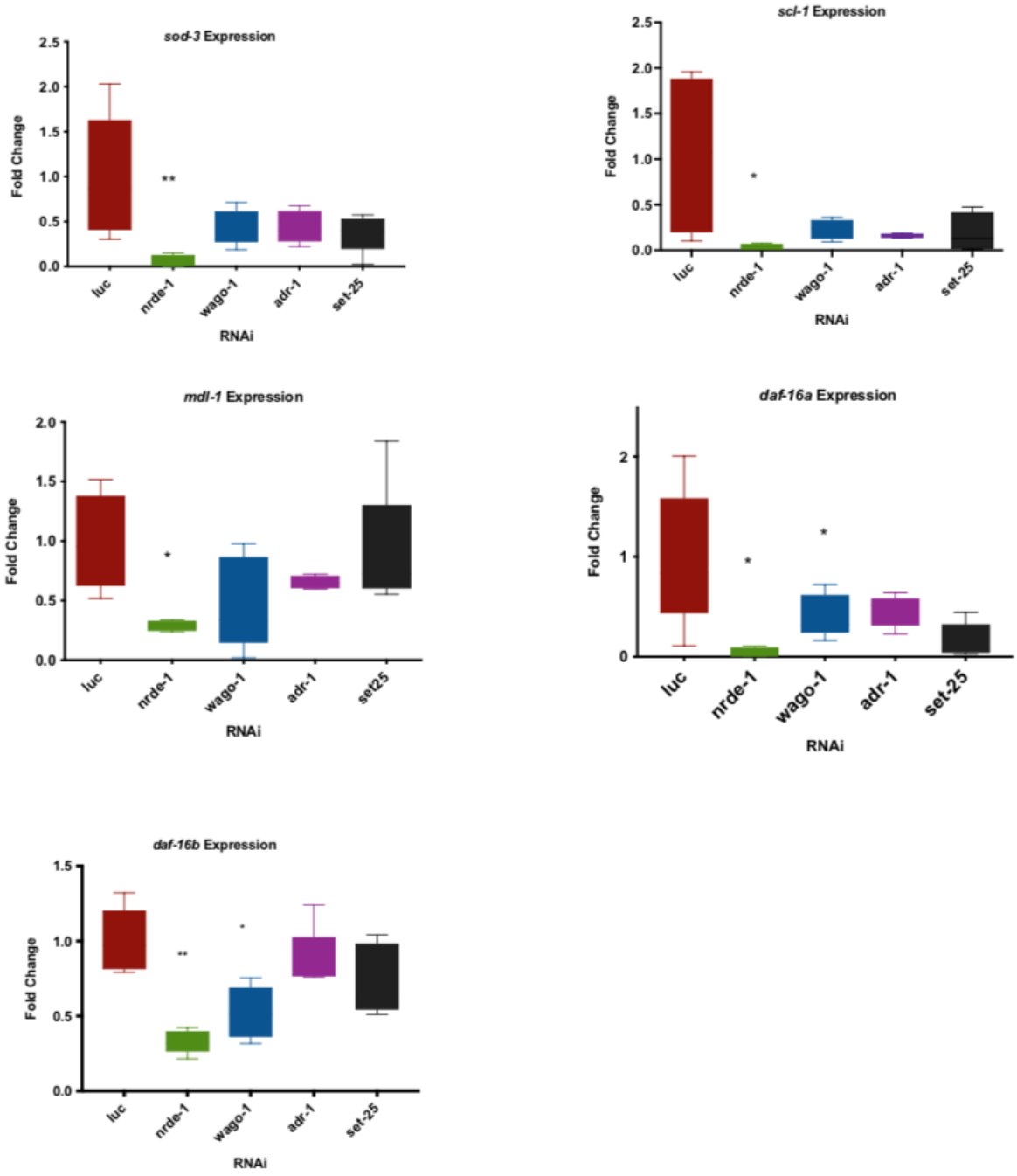


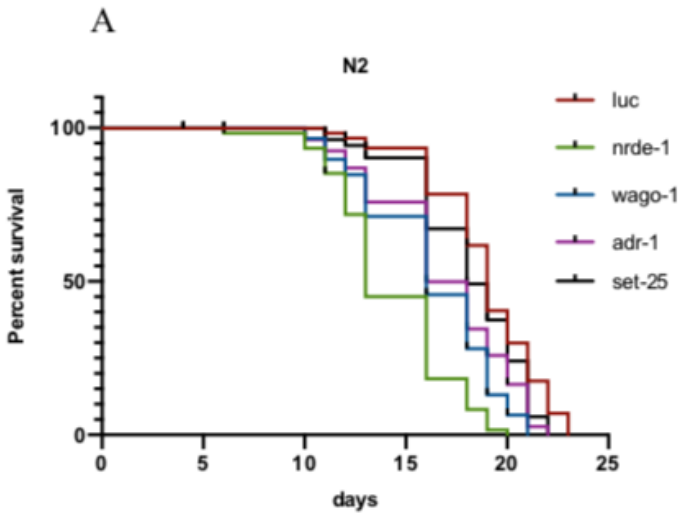
Figure 9. DAF-16 target genes; e.g. *sod-3,scl-1,mdl-1, daf-16a, daf-16b* measured by RT-qPCR to assess endogenous transcription activity of DAF-16. *nrde-1, wago-1, adr-1, set-25* genes were silenced and luciferase was used as a control. Number of replicates= 5. $*p < 0.05$; $**p < 0.01$. Two-tailed student *t* test was used for comparison with the control.

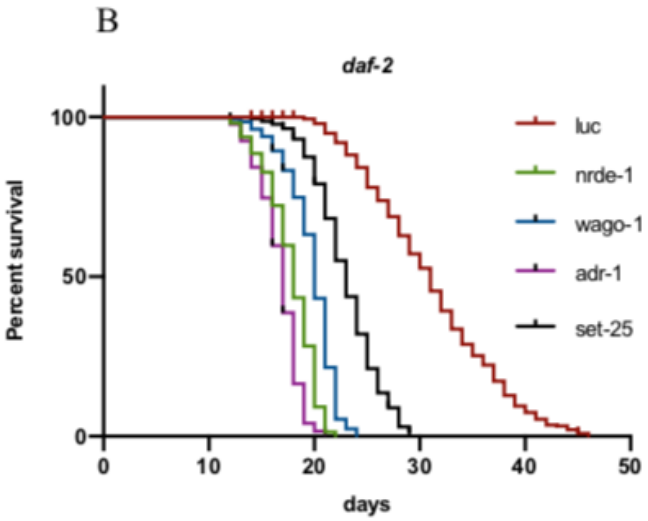
4.2. The candidate genes (*nrde-1, wago-1, adr-1* and *set-25*) have significant impact on *C. elegans* lifespan.

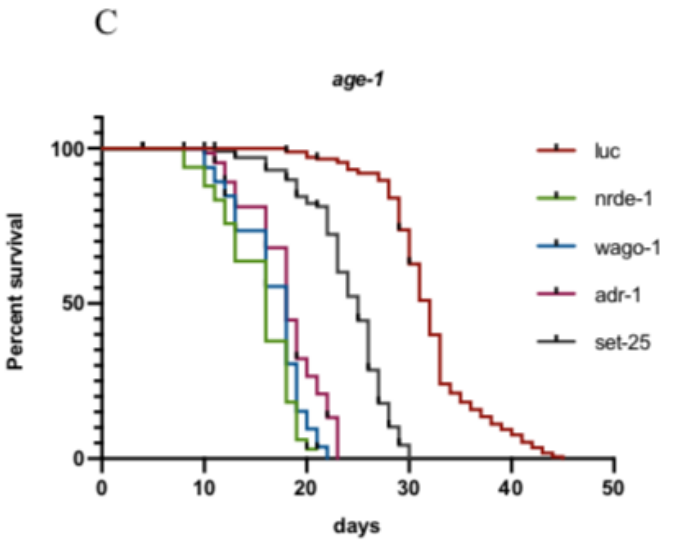
It is already known that DAF-16 expression can control development and aging in nematode *C. elegans*. Once we found that the candidate genes affect endogenous DAF-16 function, we then decided to verify if they would be involved in controlling lifespan.

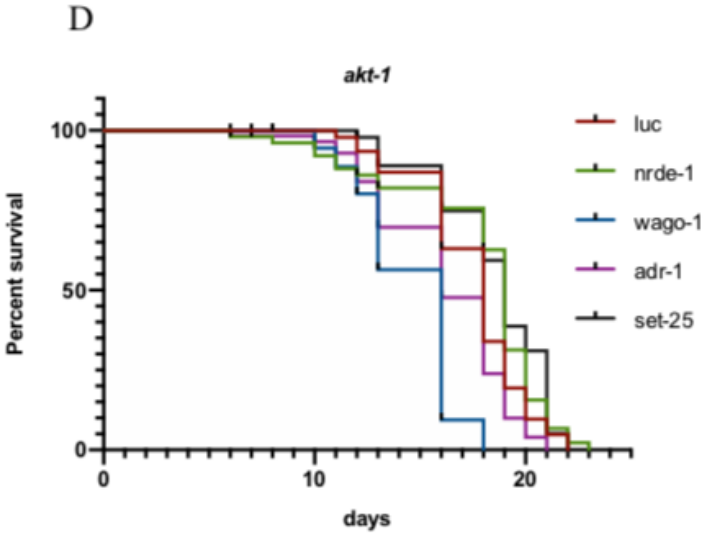
To evaluate this, we performed lifespan experiment testing if knocking down *nrde- 1, wago-1, adr-1* and *set-25* in either wild-type or *daf-2* mutant is sufficient to reduce lifespan. In parallel, we also performed lifespan assays on mutants for other members of the same pathway, i.e. *pdk-1, akt-1,* and, *age-1*. Another important question is whether the candidate genes affect lifespan by a manner dependent on DAF-16. Whether the answer to that question is yes, we expect that the lifespan of the worms treated with RNAi against the candidate genes in a *daf- 16* mutant background will be equal to the lifespan of *daf-16* mutant worms alone (Figure 10).

Interestingly, we could see a decline in the lifespan of WT and a complete suppression of longevity induced by *daf-2, pdk-1*, and *age-1* mutations when the candidate genes were silenced. They seemed to have a significant impact, especially NRDE-1, ADR-1 and WAGO-1. But, in case of *akt-1*, there was a slight increase in the lifespan when NRDE-1 was silenced in comparison to the control RNAi (LUCIFERASE), suggesting that *akt-1* loss of function suppresses the short lifespan resulting of *nrde-1* RNAi.


A
N2
Percent survival
days
luc
nrde-1
wago-1
adr-1
set-25


B
daf-2
Percent survival
days
luc
nrde-1
wago-1
adr-1
set-25


C
age-1
Percent survival
days
luc
nrde-1
wago-1
adr-1
set-25


D
akt-1
Percent survival
days
luc
nrde-1
wago-1
adr-1
set-25

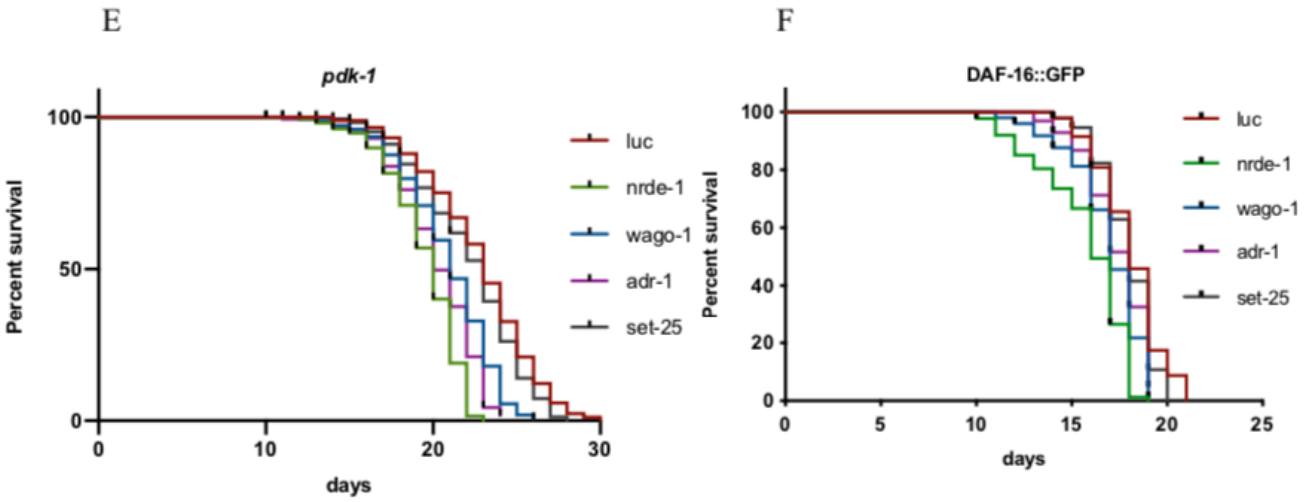


Figure 10. Lifespan analysis. *nrde-1, wago-1, adr-1, set-25* genes were silenced and luciferase was used as a control. Data was compared using the log-rank (Mantel- Cox) test. The statistics is available in table S1 (appendix).

A. N2 (Wild-type)

B. *daf-2 (e1370)*

C. *age-1 (hx546) II*

D. *akt-1 (mg144)*

E. MAM71 (DAF-16::GFP)

4.3. Why *akt-1* loss of function suppressed the lifespan of *nrde-1* RNAi?

To understand the matter, we decided on measuring the expression of the members of the IIS pathway itself - *akt-1, age-1, pdk-1* and *daf-18* - by RT-qPCR in N2 (wild-type) and *daf-2* mutants. In this case, the worms were maintained at 20°C until L2 and then transferred to 25°C and the genes *nrde-1* and *daf-16* were silenced by RNAi feeding mechanism and luciferase was used as a control (Figure 11).

We then put up a comparison graph of N2 and *daf-2* mutant from which it becomes evident that *daf-2* mutation induces by many folds the expression of these genes, consistent with a compensatory upregulation when the pathway is not active. Interestingly, this upregulation doesn't seem to be mediated by DAF- 16, as it is not inhibited by *daf-16* RNAi. Also, *nrde-1* RNAi did not affect gene expression, suggesting it does not act by promoting the expression of components of the IIS pathway.

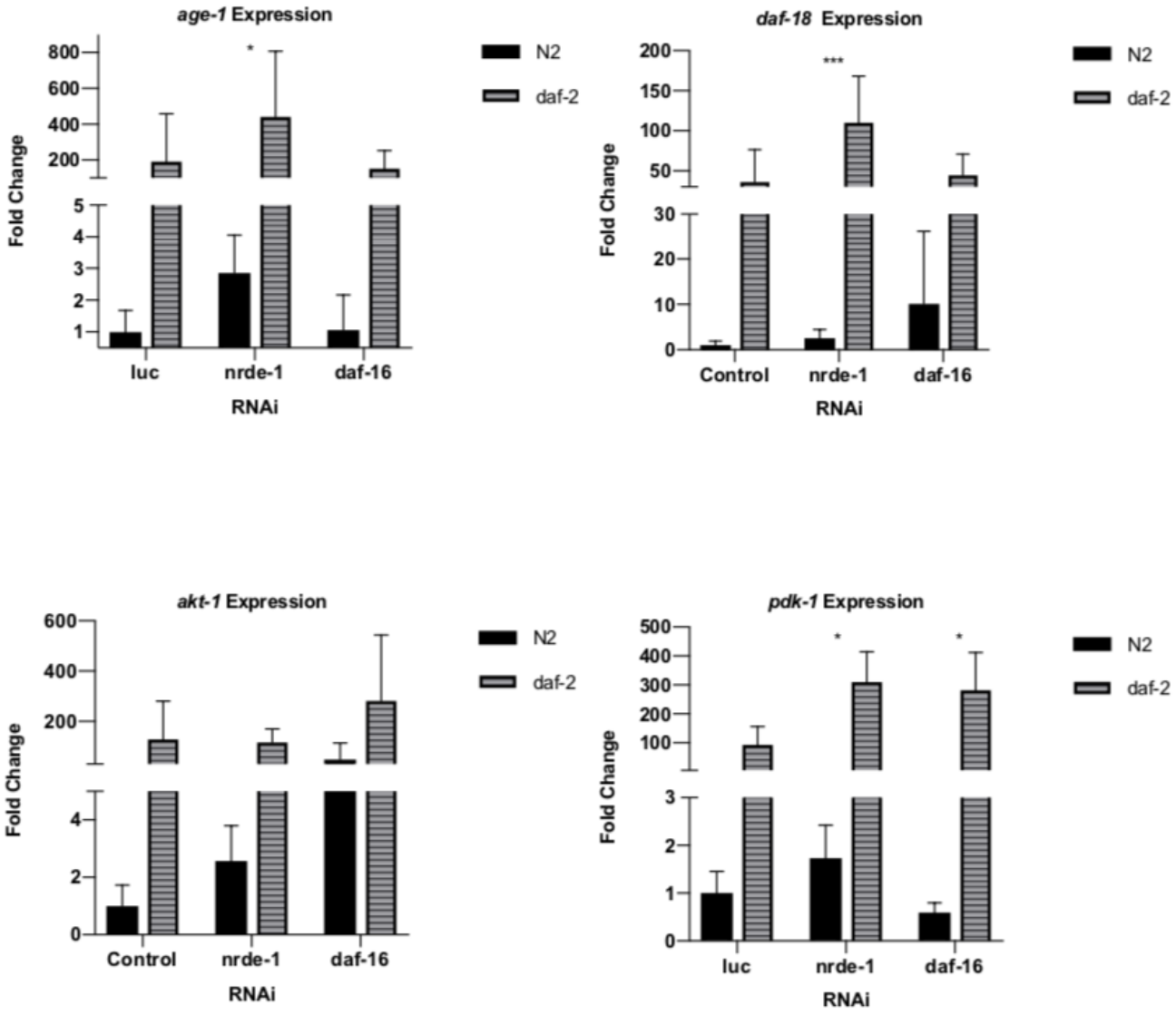


Figure 11. A comparison of *pdk-1*, *akt-1*, *age-1* and *daf-18* expression in N2 and CB1370 *daf-2 (e1370)* mutants. *nrde-1, daf-16* genes were silenced and luciferase RNAi was used as a control. Number of replicates= 4. Two-way Anova and Bonferroni's post-test was used for comparison. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4.4. Is there an interaction between NRDE-1 and the TOR pathway?

Given that *nrde-1* RNAi not only supresses *age-1* mutant-induced longevity but also reduces it when compared to the wild type worms, we first proposed that NRDE-1 might interact with a component of the IIS downstream of AGE-1/PI3K. Moreover, since *akt-1* mutation rescues the short lifespan of *nrde-1* RNAi, we hypothesized that NRDE-1 is acting through AKT-1. Our hypothesis was that somehow *nrde-1* loss of function would be leading to activation of AKT-1 independent of DAF-2/AGE- 1 signalling.

AKT-1, also referred to as protein kinase B, is one of the three isoforms of the serine/threonine kinase AKT family, which regulates metabolism, signalling, cell survival, proliferation, and growth [84]. IIS signaling mediates the phosphorylation of AKT-1. The PI3K converts PIP2 to PIP3, which then recruits AKT- 1 and PDK-1 to the membrane. PDK-1 phosphorylates AKT-1 at Thr-308 and in a parallel signaling cascade, activated mTORC2 phosphorylates AKT-1 at Ser-473 [85]. *rict-1* encodes the *C. elegans* ortholog of mammalian Rictor, a component of the target of rapamycin complex 2 (TORC2). In *C. elegans*, *rict-1* is involved in the regulation of fat metabolism, nutrition, growth, and life span [86]. Moreover, it has been observed that the high-fat phenotype of rictor mutants are genetically dependent on *akt-1, akt-2,* and serum and glucocorticoid-induced kinase-1 (*sgk-1*) [87] [88].

We therefore tested whether *nrde-1* RNAi requires *rict-1* to reduce lifespan and reverse longevity induced by the *daf-2* mutation and to do this we used a double- RNAi feeding scheme (i.e., knockdown of *akt-1:nrde-1, rict-1:nrde-1, rict-1:akt- 1*) combined with single RNAi and checked the DAF- 16::GFP translocation on *daf-2* mutant worms expressing the DAF- 16::GFP and wild type worms expressing the same transgene (Figure 12-13).

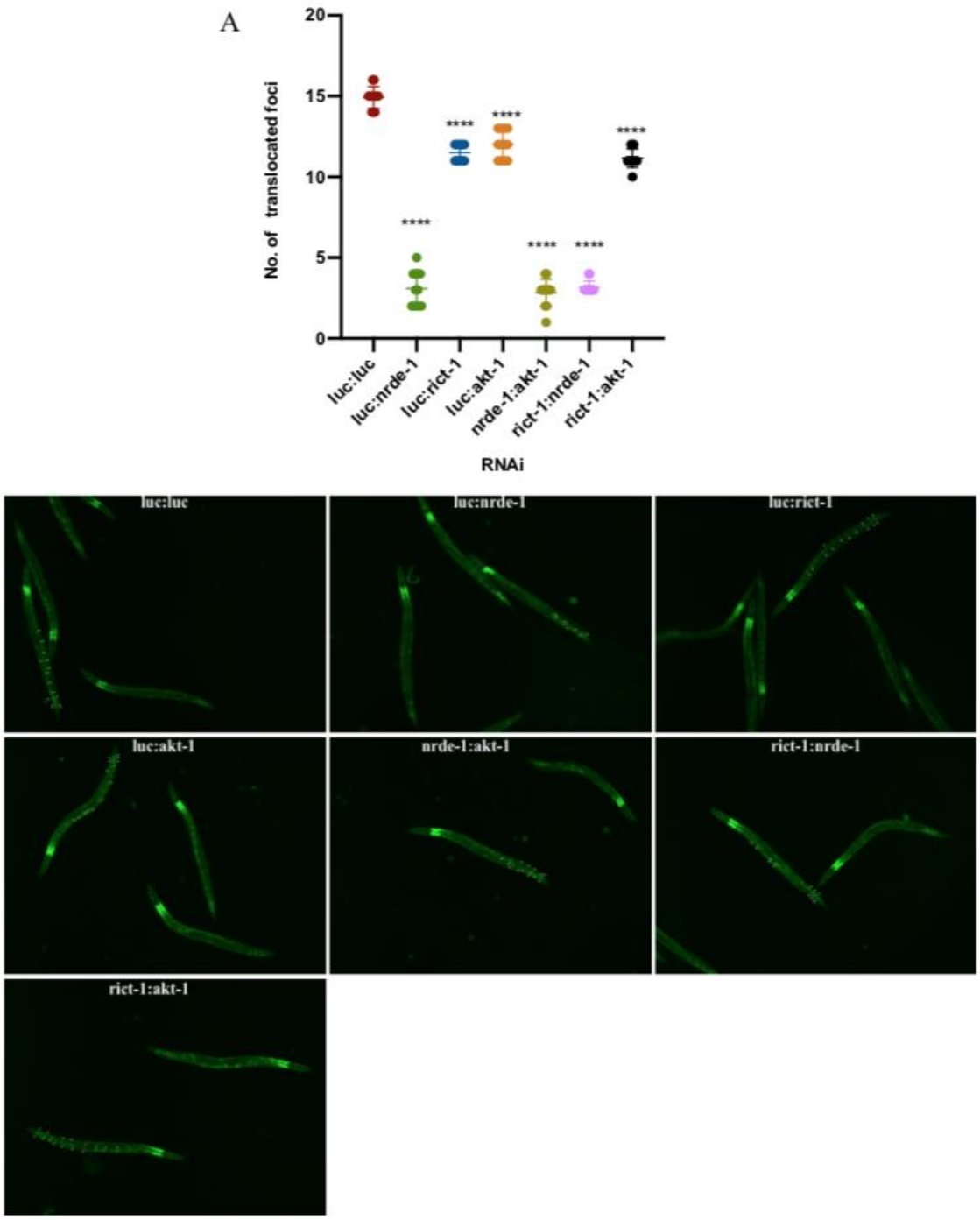


Figure 12. DAF-16 translocation assay in heat stressed MAM71 DAF-16::GFP worms. The number of DAF-16::GFP positive nucleus was scored using Cytation V. *luc:nrde-1, luc:rict-1, luc:akt-1, nrde-1:akt-1, rict-1:nrde-1* and *rict-1:akt-1* RNAi were used. The experiments were performed three times with at least 10-12 worms per group each time. **p* < 0.0001, compared to '*luc:luc* (control)' using one-way ANOVA and Tukey's multiple comparison post-hoc test. MAM71 worms were maintained at 20°C and heat-shocked at 34°C for 1.5 hours on the first day of adulthood, N=13.

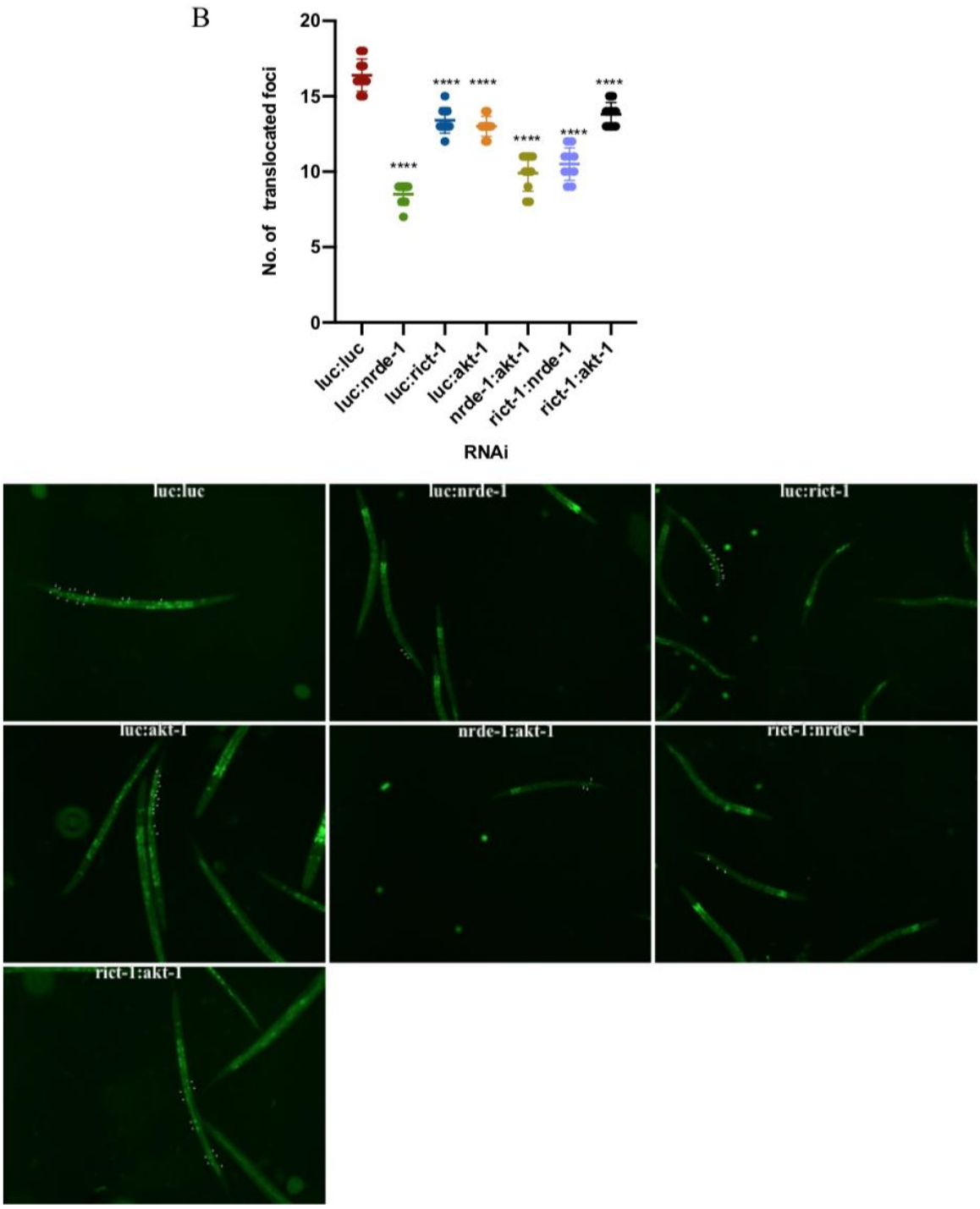


Figure 13. DAF-16 translocation assay in CF2570 *[daf-16(mu86) I; daf-2(e1370) III; muIs142 (ges-1p::GFP::daf-16(cDNA) + odr-1p::RFP)]* worms. The number of DAF-16::GFP positive nucleus was scored using Cytation V. *luc:nrde-1, luc:rict-1, luc:akt-1, nrde-1:akt-1, rict-1:nrde-1* and *rict-1:akt-1* RNAi were used. The experiments were performed three times with at least 10-12 worms per group each time.*$p < 0.0001$, compared to '*luc:luc* (control)' using one-way ANOVA and Tukey's multiple comparison post-hoc test. CF2570 worms were maintained at 15°C till L2 and then transferred to 25°C, N=12.

As expected, in case of *luc:nrde-1* we could see a significant down-regulation of DAF-16::GFP translocation. A small down-regulation was observed in *luc:rict- 1, luc:akt-1* and *rict-1:akt-1* when compared to the control (*luc:luc*). But unexpectedly, *nrde-1:akt-1* and *rict-1:nrde-1* did not up-regulate the translocation, from which it becomes evident that NRDE-1 doesn't interact with the TORC2 pathway to activate AKT-1 independent of DAF-2/AGE-1 signalling.

4.5. Is there an interaction between NRDE-2, NRDE-3, NRDE-4 and the IIS pathway?

4.5.1. Effects of *nrde-1, nrde-2, nrde-3* and *nrde-4* knock-down on:

4.5.1.1. DAF-16 Translocation

To test whether other members of the NRDE pathway were involved in DAF-16 translocation, we silenced *nrde-1, nrde-2, nrde-3* and *nrde-4* using the RNAi feeding mechanism in *daf-2* mutant worms expressing the DAF-16::GFP construct and wild type worms expressing the same transgene. We therefore monitored the DAF-16::GFP localization in L4 worms (Figure 14-15).

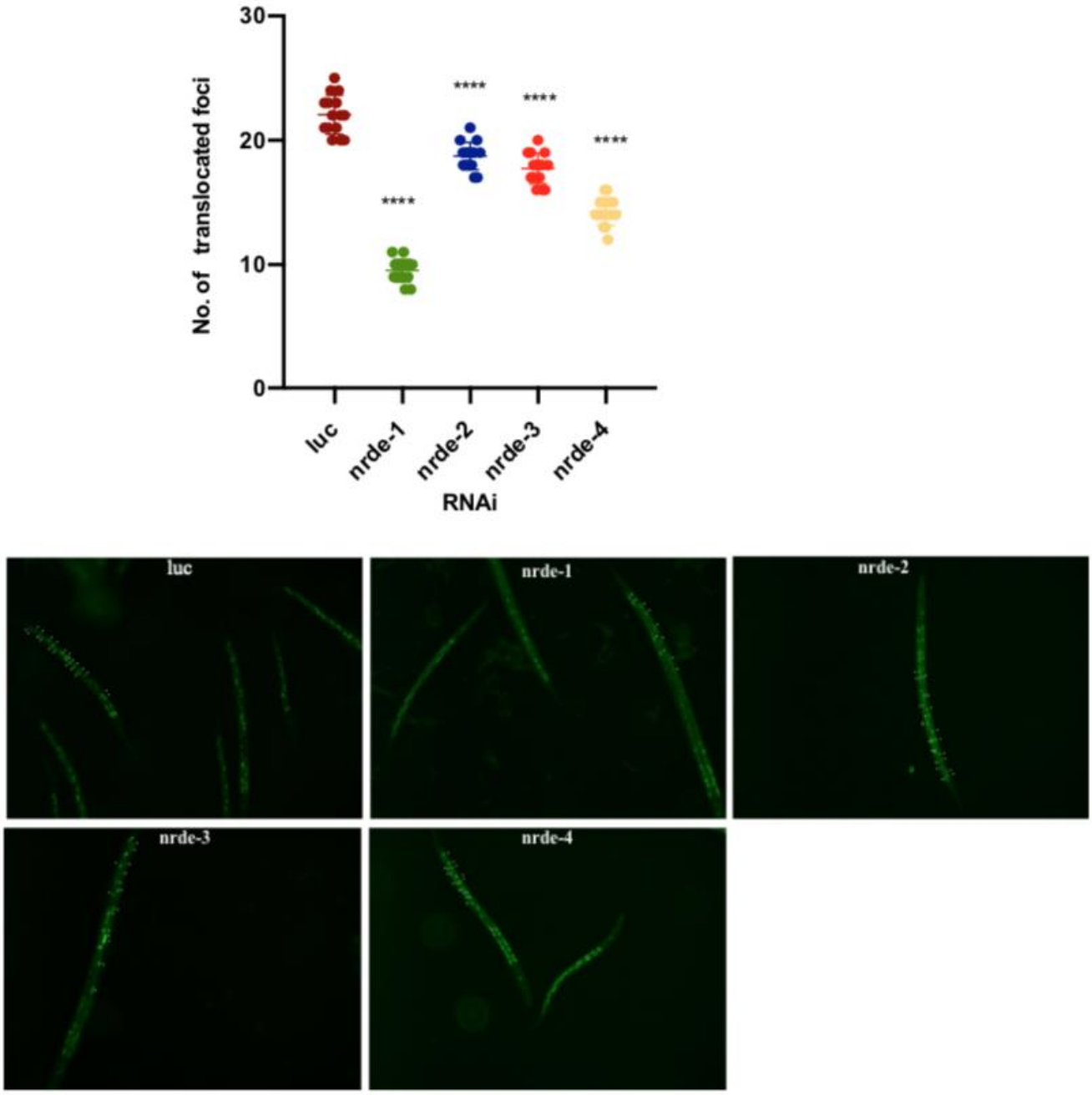


Figure 14. DAF-16 translocation assay in heat stressed MAM71 DAF-16::GFP worms. The number of DAF-16::GFP positive nucleus was scored using Cytation V. *nrde-1, nrde-2, nrde-3, nrde-4* genes were silenced and luciferase was used as a control. The experiments were performed three times with at least 10-15 worms per group each time, ****$p < 0.0001$. Two-tailed student *t*-test was used for comparison with the control (*luciferase*). Representative images of DAF-16 translocation are below each graph. MAM71 were maintained at 20°C and heat-shocked at 34°C for 1.5 hours on the first day of adulthood, N=12.

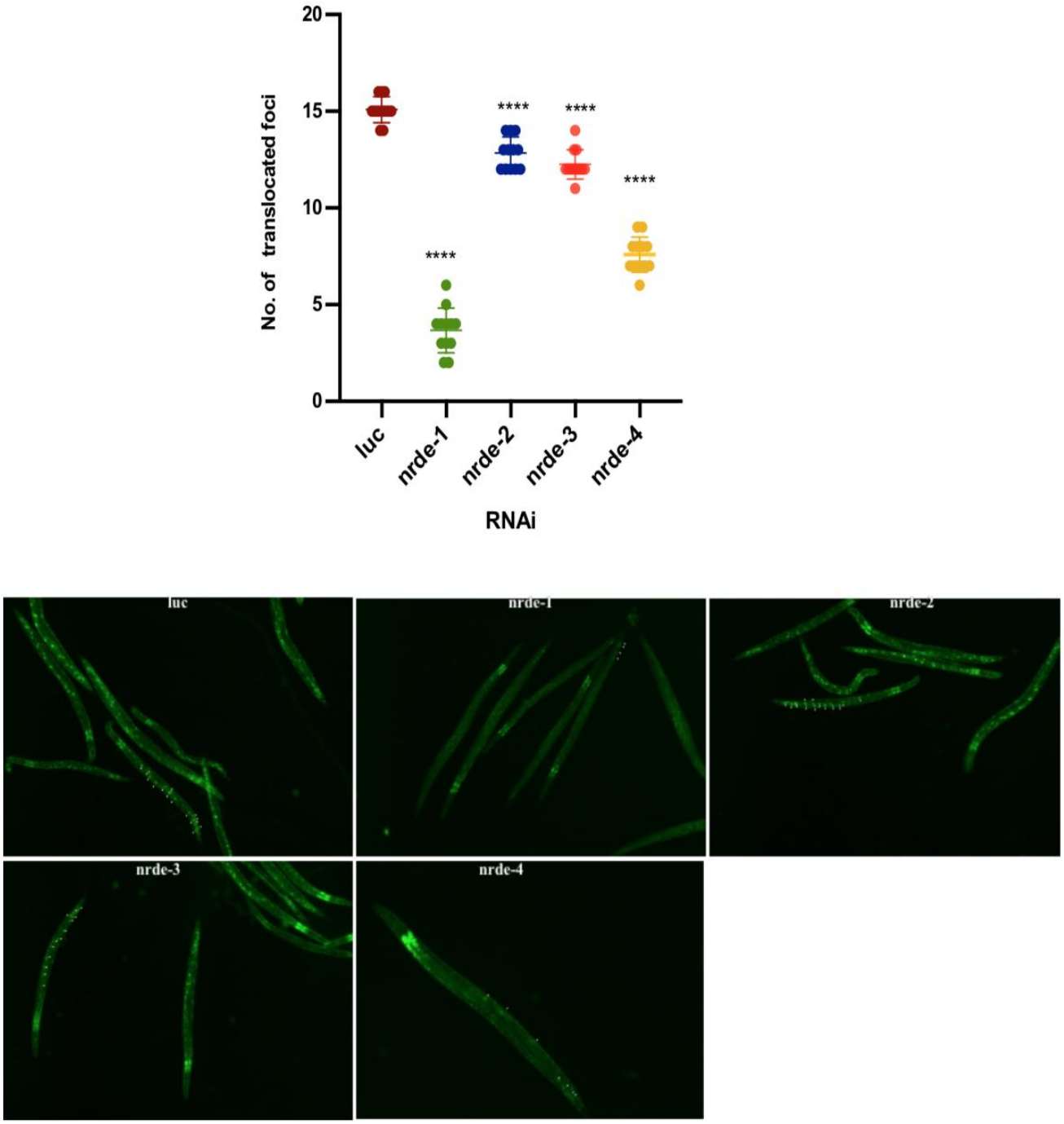


Figure 15. DAF-16 translocation assay in CF2570 *[daf-16(mu86) I; daf-2(e1370) III; muIs142 (ges-1p::GFP::daf-16(cDNA) + odr-1p::RFP)]* worms. The number of DAF-16::GFP positive nucleus was scored using Cytation V. *nrde-1, nrde-2, nrde-3, nrde-4* genes were silenced and luciferase was used as a control. The experiments were performed three times with at least 10-15 worms per group each time, ****$p < 0.0001$. Two-tailed student *t*-test was used for comparison with the control (*luciferase*). Representative images of DAF-16 translocation are below each graph. CF2570 worms *were* maintained at 15°C till L2 and then transferred to 25°C, N=15.

The observation was that along with NRDE-1 somehow NRDE-4 also inhibits DAF-16::GFP nuclear localization. Although NRDE-2 and NRDE-3 seem to decrease the nuclear localization to some extent, the impact was not so significant as compared to the other two NRDEs. These results suggest that the recruitment of NRDE-1/NRDE-4 to the chromatin is particularly important for DAF-16 function and this somehow works independently of NRDE-2 and -3 and potentially endo-siRNAs.

4.5.1.2. *Dauer* formation

To test the dependence of *nrde-1, nrde-2, nrde-3* and *nrde-4* on the *dauer* formation, we silenced these genes in *daf-2* mutants and evaluated the presence of the *dauer* stage in the population. The worms were maintained at 25°C since eggs and they were scored for the presence of *dauer* and non-*dauer* under the microscope after 60-65 hours. Consistent with DAF-16 translocation, silencing of the candidate genes, particularly *nrde-1* and *-4*, resulted in a decrease of *dauer* population (Figure 16).

Therefore, this result suggests that knocking-down the candidate genes suppresses the *dauer* arrest phenotype of *daf-2*, which suggests that the genes are major regulators of endogenous DAF-16.

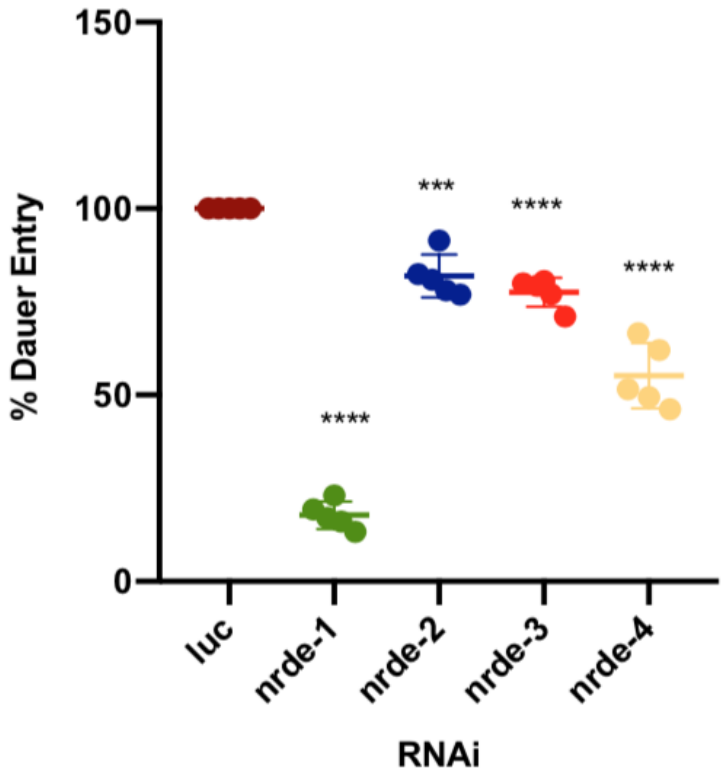


Figure 16. Dauer assay of *daf-2(e1370)* mutants. *nrde-1*, *nrde-1*, *nrde-3*, *nrde-4* genes were silenced and luciferase was used as a control. N=100 and number of replicates= 5. ***p < 0.001; ****p < 0.0001. Two-tailed student *t*-test was used for comparison with the control (*luciferase*).

4.5.1.3. Lifespan

We performed lifespan experiment knocking-down *nrde-2*, *nrde-3* and *nrde-4* to check if they have a similar impact as *nrde-1* on either wild-type or *daf-2* mutants (Figure 17).

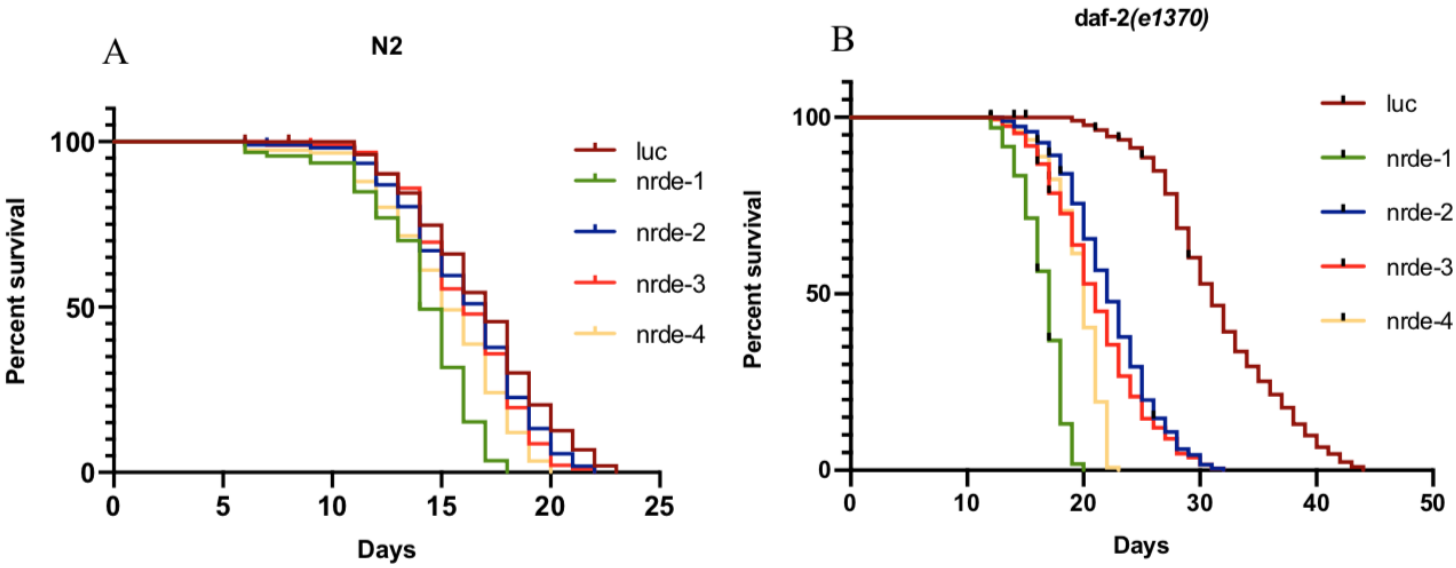


Figure 17. Lifespan analysis. *nrde-1, nrde-2, nrde-3, nrde-4* genes were silenced and luciferase was used as a control. Data was compared using the log-rank (Mantel- Cox) test. The statistics is available in table S2 (appendix).

A. N2 (Wild-type)

B. *daf-2 (e1370)*

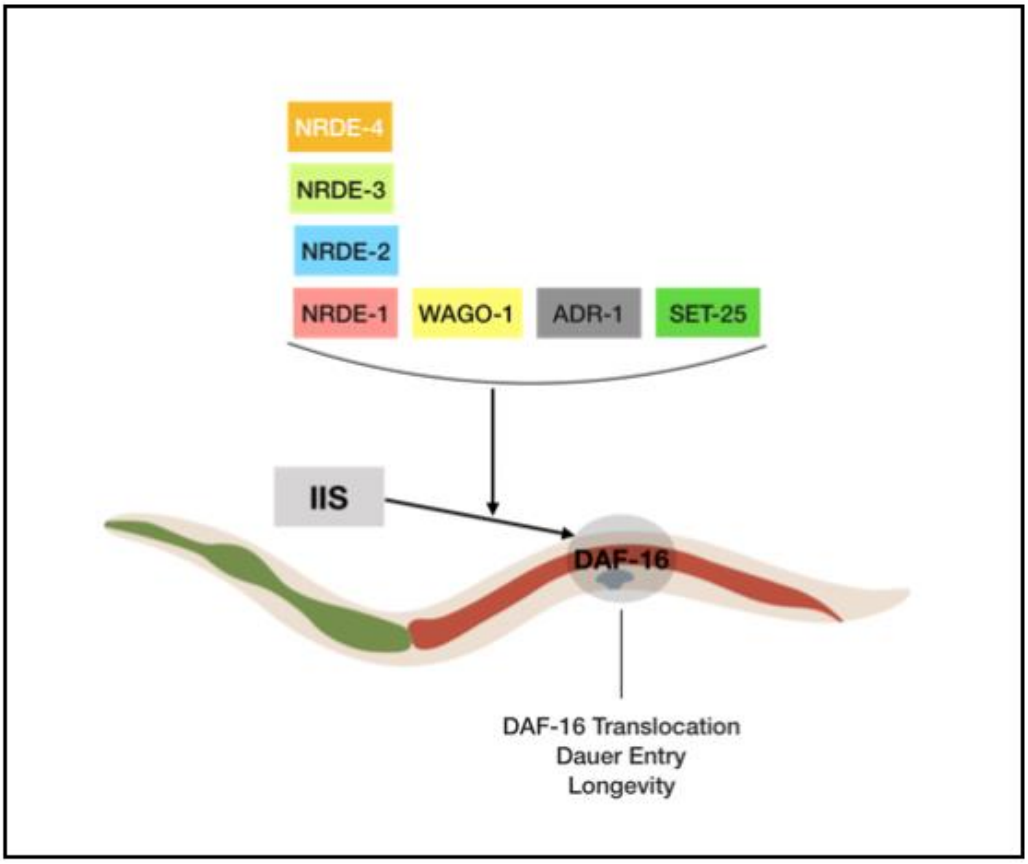
Interestingly, we could see a strong decline in the lifespan of WT when NRDE-1 and NRDE-4 were silenced and a suppression of longevity induced by *daf-2* when the candidate genes were silenced.

5. CONCLUSION AND FUTURE DIRECTIONS

This study demonstrates that ADR-1, SET-25, WAGO-1, and especially NRDE-1 inhibit DAF-16 nuclear localisation and DAF- 16-induced *dauer* entry and longevity. We can conclude that these proteins are interacting at some level with the IIS to regulate DAF-16 function and longevity. In light of the data, our hypothesis was that NRDE- 1 interacts with a component of the IIS downstream of AGE-1/PI3K, i.e. AKT-1, by interacting with the TORC2 pathway, although the results indicate that NRDE-1 doesn't act through the TORC2 pathway in activating AKT-1 (Figure 18-19).

We could also demonstrate that the other NRDEs (NRDE-2, NRDE-3, NRDE-4) have interactions with the IIS pathway at the level of DAF- 16::GFP translocation, *dauer* formation and lifespan regulation (Figure 18), although the interactions of NRDE-2 and -3 were less so than of NRDE-1 and -4. In the future, it will very tempting to speculate why NRDE-2 and NRDE-3 don't have the same pattern as the other two.

This entire study suggests that epigenetic modifiers such as NRDE-1 are strong regulators of DAF-16/FOXO function, and therefore play a role in *dauer* formation and longevity in *C. elegans*.



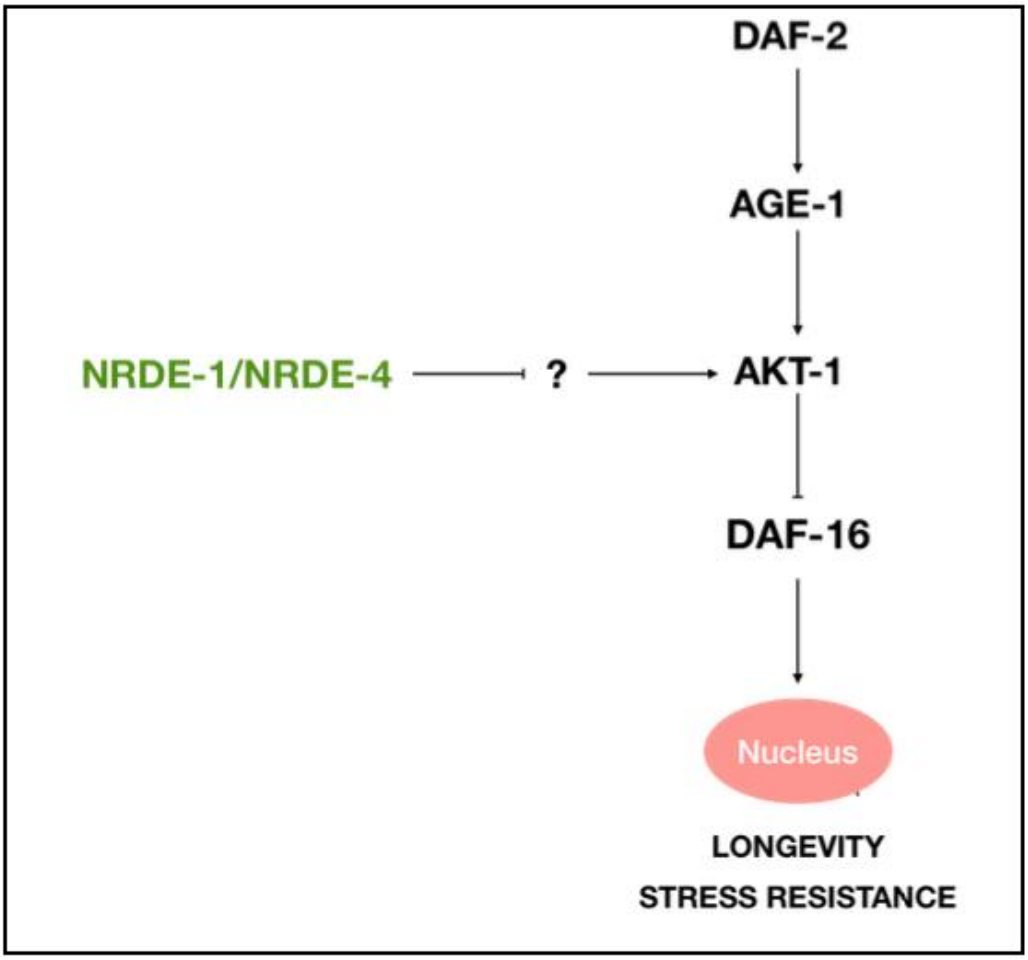


Figure 18-19. Current model

A recent study shows that DAF-16, apart from being targeted by AKT kinase, is also being targeted by the other two kinases; TAX-6•CNB-1 and UNC-43, the *C. elegans* Calcineurin and Ca (2+)/calmodulin-dependent kinase type II (CAMKII) orthologs, respectively. CAMKII, which is not involved in the IIS pathway, generally targets DAF-16 when the worms are exposed to heat, stress, or starvation [89].

An interesting point to note: unlike AKT-1 deactivating DAF-16's entry into the nucleus, CAMKII phosphorylates DAF-16 at the S286 site and promotes its nuclear localization. Calcineurin, in turn, neutralizes this act by removing the phosphate group, rendering the sequestration of DAF-16 in the cytosol [89].

6. BIBLIOGRAPHY

[1] P. L. Fontana L., " Promoting health and longevity through diet: from model organisms to humans," *Cell.* , vol. 161, p. 106–118, 2015.

[2] J. T. Friedman DB, "A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility.," *Genetics.* , vol. 118, no. 1, pp. 75-86, 1988 .

[3] Kimura et al., "daf-2, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*.," *Science,* vol. 277, no. 5328, pp. 942-6, 1997.

[4] Kenyon et al., "A C. elegans mutant that lives twice as long as wild type.," *Nature.* , vol. 366, no. 6454, pp. 461-4 , 1993.

[5] Brenner S., " The genetics of Caenorhabditis elegans.," *Genetics.* , vol. 77, no. 1, pp. 71-94, 1974.

[6] Fire et al., "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans," *Nature,* vol. 391, pp. 806-811, 1998.

[7] M. Chalfie, "GFP: Lighting up life," *Proc Natl Acad Sci U S A,* vol. 106, no. 25, p. 10073–10080, 2009.

[8] Gems et al., "Defining wild-type life span in *Caenorhabditis elegans*.," *J. Gerontol. A Biol. Sci. Med. Sci* , vol. 55, p. B215–B219, 2000.

[9] Altun et al., "Handbook of *C. elegans* Anatomy.In WormAtlas," 2019. [Online].Available:http://www.wormatlas.org/hermaphrodite/hermaphroditehomepage.htm . [Accessed 18 November 2019].

[10] Gruber et al., "Deceptively simple but simply deceptive –*Caenorhabditis elegans* lifespan studies: Considerations for aging and antioxidant effects.," *FEBS Letters,* vol. 583, no. 21, pp. 3377-3387, 2009.

[11] Sulston et al., "Post-embryonic cell lineages of the nematode *Caenorhabditis elegans*.," *Dev. Biol. ,* vol. 56, pp. 110-156, 1977.

[12] Sulston et al., "The *Caenorhabditis elegans* male: Postembryonic development of nongonadal structures.," *Dev Biol. ,* vol. 78, pp. 542-576 , 1980.

[13] Nguyen et al., "Morphogenesis of the *Caenorhabditis elegans* male tail tip.," *Dev. Biol. ,* vol. 207, pp. 86-106, 1999.

[14] K. a. S. P. Liu, "Sensory regulation of male mating behavior in *Caenorhabditis elegans*," *Neuron ,* vol. 14, pp. 79-89, 1995.

[15] Garcia et al., " Regulation of distinct muscle behaviors controls the *C. elegans* male's copulatory spicules during mating," *Cell ,* vol. 107, pp. 777-788, 2001.

[16] Anderson et al., "Outcrossing and the Maintenance of Males within C. elegans Populations," *J Hered. ,* vol. 101, p. S62–S74, 2010.

[17] Meneely et al., "Meiotic Mutants That Cause a Polar Decrease in Recombination on the X Chromosome in *Caenorhabditis elegans*," *Genetics ,* vol. 136, pp. 119-127, 1994.

[18] J. Hodgkin, "Sexual dimorphism and sex determination," in *In The nematode C. elegans (ed. W.B. Wood). Chap. 9.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988, pp. 243-279.

[19] R. D. Golden JW, " The Caenorhabditis elegans dauer larva: developmental effects of pheromone, food, and temperature.," *Dev Biol. ,* vol. 102, no. 2, pp. 368-78, 1984.

[20] R. D. Golden JW, "A pheromone influences larval development in the nematode *Caenorhabditis elegans*.," *Science. ,* vol. 218, no. 4572, pp. 578- 80, 1982.

[21] Ruzanov et al., "Genes that may modulate longevity in C. elegans in both dauer larvae and long-lived daf-2 adults," *Exp Gerontol.* , vol. 42, no. 8, p. 825–839, 2007.

[22] Shaw et al., "The *C.elegans* TGF-bDauer Pathway Regulates Longevity via Insulin Signaling," *Current Biology,* vol. 17, p. 1635–1645, 2007.

[23] Lin et al., " daf-16: An HNF-3/forkhead family member that can function to double the life-span of C. elegans.," *Science* , vol. 278, p. 1319–1322, 1997.

[24] Ogg et al., "The Fork head transcription factor DAF-16 transduces insulin- like metabolic and longevity signals in *C. elegans*," *Nature* , vol. 389, p. 994–999, 1997.

[25] J. T. Friedman DB, "A mutation in the age-1 gene in *Caenorhabditis elegans* lengthens life and reduces hermaphrodite fertility," *Genetics.* , vol. 118, no. 1, pp. 75-86 , (1988).

[26] Riddle et al., " Interacting genes in nematode dauer larva formation," *Nature* , vol. 290, pp. 668-671, 1981.

[27] Kenyon CJ, "The genetics of ageing.," *Nature,* vol. 464, no. 7288, pp. 504- 512, (2010).

[28] P. L. L. V. Fontana L, "Extending healthy life span--from yeast to humans," *Science* , vol. 328, no. (5976), pp. 321-6, 2010.

[29] Sun et al., " DAF-16/FOXO Transcription Factor in Aging and Longevity," *Front. Pharmacol.,* vol. 8, no. 548, 2017.

[30] Kenyon CJ, "The genetics of ageing.," *Nature,* vol. 464, no. 7288, pp. 504- 512, (2010).

[31] Partridge et al., "Forkhead transcription factors and ageing," *Oncogene* , vol. 27, p. 2351–2363, 2008.

[32] Lin et al., "daf-16: An HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans.*," *Science.* , vol. 278, no. (5341), pp. 1319-22, 1997.

[33] Ogg et al., "The Fork head transcription factor DAF-16 transduces insulin- like metabolic and longevity signals in *C. elegans*.," *Nature,* vol. 389, no. (6654), pp. 994-9, 1997.

[34] Yen et al., "DAF-16/Forkhead Box O Transcription Factor: Many Paths to a Single Fork(head) in the Road.," *Antioxid Redox Signal,* vol. 14, no. 4, p. 623–634, 2011.

[35] Dorman et al., "The age-1 and daf-2 genes function in a common pathway to control the lifespan of *C. elegans.*," *Genetics ,* vol. 141, no. 4, pp. 1399- 1406, (1995).

[36] Paradis et al., " A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in *C. elegans*," *Genes Dev ,* vol. 13, no. 11, p. 1438–1452, (1999).

[37] Zhou et al., "(1998) Disruption of Dictyostelium PI3K genes reduces [32P]phosphatidylinositol 3,4 bisphosphate and [32P]phosphatidylinositol trisphosphate levels, alters F-actin distribution and impairs pinocytosis," *J Cell Sci ,* vol. 111, p. 283–294, (1998).

[38] R. G. Paradis S, " *C. elegans* Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor," *Genes Dev,* vol. 12, no. 16, p. 2488–2498, (1998).

[39] C. T. &. H. P. J. Murphy, "Insulin/insulin-like growth factor signaling in *C. elegans*," *WormBook ,* pp. 1-43, (2013).

[40] D. J. e. a. Clancy, "Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein," *Science ,* vol. 292, p. 104–106 , (2001).

[41] Tatar et al., "A mutant Drosophila insulin receptor homolog that extends lifespan and impairs neuroendocrine function," *Science ,* vol. 292, pp. 107- 110, (2001).

[42] Blüher et al., "Extended longevity in mice lacking the insulin receptor in adipose tissue.," *Science ,* vol. 299, no. 5606, pp. 572-4, 2003.

[43] Holzenberger et al., "IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice," *Nature* , vol. 421, no. 6919, pp. 182-7, 2003.

[44] Brown-Borg et al., " A. Dwarf mice and the ageing process," *Nature,* vol. 384, pp. 33-33, (1996).

[45] Flurkey et al., "Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production," *Proc. Natl. Acad. Sci.* , vol. 98, p. 6736–6741, (2001).

[46] Brown-Borg et al., " Dwarf mice and the ageing process.," *Nature 384, 33,* vol. 384, no. 6604, 1996.

[47] M. e. a. Kuro-o, "Mutation of the mouse klotho gene leads to a syndrome resembling ageing," *Nature,* vol. 390, p. 45–51 , (1997).

[48] H. Kurosu, "Suppression of Aging in Mice by the Hormone Klotho.," *Science (80-.)* , vol. 309, p. 1829–1833 , (2005).

[49] Suh et al., "Functionally significant insulin-like growth factor I receptor mutations in centenarians," *Proc Natl Acad Sci U S A* , vol. 105, p. 3438– 3442 , (2008).

[50] Willcox et al., "FOXO3A genotype is strongly associated with human longevity," *Proc. Natl Acad. Sci. USA* , vol. 105, p. 13987–13992, (2008).

[51] Cantó et al., " AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity", *Nature,* vol. 458, p. 1056–1060, 2009.

[52] X Sun et al., " DAF-16/FOXO transcription factor in aging and longevity," *Frontiers in Pharmacology,* vol. 8, 2017.

[53] Greer et al., "AMP-activated protein kinase and FoxO transcription factors in dietary restriction-induced longevity," *in Annals of the New York Academy of Sciences* , vol. 1170, p. 688–692 , (2009).

[54] Inoki et al., " TSC2 mediates cellular energyresponse to control cell growth and survival," vol. 115, p. 577–590, 2003.

[55] Zhou et al., " Longevity and stress in *Caenorhabditis elegans*," *Aging*, vol. 3, p. 733–753 , (2011).

[56] Tullet et al., "Direct Inhibition of the Longevity-Promoting Factor SKN-1 by Insulin-like Signaling in *C. elegans*," *Cell* , vol. 132, p. 1025–1038 , (2008).

[57] M. J. e. a. Steinbaugh, "Lipid-mediated regulation of SKN-1/Nrf in response to germ cell absence," *Elife* , vol. 4, (2015).

[58] Washburn &. Hundley, "Trans and cis factors affecting A-to-I RNA editing efficiency of a noncoding editing target in *C. elegans*," *RNA* , vol. 22, p. 722–8 , (2016).

[59] Tonkin et al., " RNA editing by ADARs is important for normal behavior in *Caenorhabditis elegans*," *EMBO J.* , vol. 21, p. 6025–6035 , (2002).

[60] Serio et al., "[PSI+]: an epigenetic modulator of translation termination efficiency," *Annu Rev Cell Dev Biol*, vol. 15, pp. 661-703., (1999).

[61] Knight et al., "The role of RNA editing by ADARs in RNAi.," *Mol Cell*, vol. 10 , pp. 809-17, (2002).

[62] J. Mitchell, "NRDE-1 is required for germline immortality," University of North Carolina at Chapel Hill Graduate School, May 2016. [Online]. Available: https://doi.org/10.17615/smvf-pq33. [Accessed November 2019].

[63] Burkhart et al., "A pre-mRNA-associating factor links endogenous siRNAs to chromatin regulation," *PLoS Genet* , vol. 7, p. e1002249, 2011.

[64] K. Reis, "Epigenetic inheritance and DNA replication in *Caenorhabditis elegans*," Universitata Pompeu Fabra, Barcelona, 2016.

[65] Ambros et al., "MicroRNAs and Other Tiny Endogenous RNAs in *C. elegans*," *Cell*, vol. 13, no. 10, pp. 807-818, 2003.

[66] B. K. K. S. Burton NO, "Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*," *Proc Natl Acad Sci U S A* , vol. 108, pp. 19683-19688., 2011.

[67] Guang et al., "Small regulatory RNAs inhibit RNA polymerase II during the elongation phase of transcription," *Nature* , vol. 465, p. 1097–1101, 2010.

[68] Shirayama et al., "The Vasa homolog RDE-12 engages target mRNA and multiple argonaute proteins to promote RNAi in *C. elegans*," *Curr. Biol.* , vol. 24, p. 845–851 , (2014).

[69] W. e. a. Gu, "Distinct Argonaute-Mediated 22G-RNA Pathways Direct Genome Surveillance in the *C. elegans* Germline," *Mol. Cell* , vol. 36, p. 231–244 , (2009).

[70] Schmitz et al., "Axon guidance genes identified in a large-scale RNAi screen using the RNAi-hypersensitive *Caenorhabditis elegans* strain nre- 1(hd20) lin-15b(hd126)." *Proc. Natl. Acad. Sci.* , vol. 104, p. 834–839, (2007).

[71] Su et al., "Muscle-Specific Histone H3K36 Dimethyltransferase SET-18 Shortens Lifespan of *Caenorhabditis elegans* by Repressing daf-16a Expression," *Cell Rep.* , vol. 22, p. 2716–2729 , (2018).

[72] Kamath et al., "Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*," *Genome Biol.* , vol. 2, (2001).

[73] Volovik et al., " Temporal requirements of heat shock factor-1 for longevity assurance," *Aging Cell.* , vol. 11, no. 3, pp. 491-9, 2012 .

[74] Ferraz et al., "IMPACT is a GCN2 inhibitor that limits lifespan in *Caenorhabditis elegans*," *BMC Biol.* , vol. 14, 2016.

[75] Ivana Bratic et al., "Mitochondrial DNA level, but not active replicase, is essential for *Caenorhabditis elegans* development," *Nucleic Acids Res.* , vol. 37, no. 6, p. 1817–1828, 2009 .

[76] Silas Pinto et al., "Enoxacin extends lifespan of *C. elegans* by inhibiting miR-34-5p and promoting mitohormesis.," *Redox Biology*, vol. 18, pp. 84- 92, 2018.

[77] Gems et al., "Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis ele*gans.," *Genetics,* vol. 150, pp. 129-55, 1998.

[78] Honda et al., "The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*," *FASEB J.* , vol. 13, pp. 1385-93, (1999).

[79] Ookuma et al. , "Identification of a DAF-16 transcriptional target gene, scl-1, that regulates longevity and stress resistance in *Caenorhabditis elegans*," *Curr Biol.* , vol. 13, no. 5, pp. 427-31, 2003 .

[80] Ookuma et al., "Identification of a DAF-16 transcriptional target gene, scl-1, that regulates longevity and stress resistance in *Caenorhabditis elegans*," *Curr Biol.* , vol. 13, no. 5, pp. 427-31, 2003 .

[81] Bansal et al., "Transcriptional regulation of *Caenorhabditis elegans* FOXO/DAF-16 modulates lifespan," *Longev Healthspan,* vol. 3, no. 5, 2014.

[82] T. R. B. A. C. M. Yuan J, "The *C. elegans* MDL-1 and MXL-1 proteins can functionally substitute for vertebrate MAD and MAX," *Oncogene,* vol. 17, no. 9, pp. 1109-18, 1998.

[83] Johnson et al., "The *Caenorhabditis elegans* Myc-Mondo/Mad ComplexesIntegrate Diverse Longevity Signals," *PLoS Genet.* , vol. 10, p. e1004278, 2014.

[84] C. L. Manning BD, "AKT/PKB signaling: navigating downstream.," *Cell,* vol. 129, no. 7, pp. 1261-74, (2007).

[85] M. Hresko RC, "mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes," *M J Biol Chem.,* vol. 280, no. 49, pp. 40406-16, 2005.

[86] Soukas et al., "Rictor/TORC2 regulates fat metabolism, feeding, growth, and life span in *Caenorhabditis elegans*," *Genes Dev.* , vol. 23, no. 4, pp. 496-511, 2009.

[87] Soukas et al., "Rictor/TORC2 regulates fat metabolism, feeding, growth, and life span in *Caenorhabditis elegans*," *Genes Dev,* vol. 23, pp. 496- 511, (2009).

[88] Jones et al., "Rictor/TORC2 regulates *Caenorhabditis elegans* fat storage, body size, and development through sgk-1," *PLoS Biol*, vol. 7, p. e60, (2009).

[89] Tao et al., "CAMKII and calcineurin regulate the lifespan of *Caenorhabditis elegans* through the FOXO transcription factor DAF-16," *eLife.* , vol. 2, no. e00518, 2013.

7. APPENDIX

Strain	Allele	RNAi	Temperature (°C)	Median lifespan (days)	N	Censored	P value vs. control
N2		luciferase	20°	19	65	7	
N2		nrde-1	20°	13	63	3	<0.0001
N2		wago-1	20°	16	62	6	<0.0001
N2		adr-1	20°	17	55	6	<0.0001
N2		set-25	20°	18	56	14	0.0003
TJ1052	*age-1*	luciferase	20°	31	178	6	
TJ1052	*age-1*	nrde-1	20°	16	68	4	<0.0001
TJ1052	*age-1*	wago-1	20°	17	68	11	<0.0001
TJ1052	*age-1*	adr-1	20°	18	68	14	<0.0001
TJ1052	*age-1*	set-25	20°	24	103	17	<0.0001
GR1310	*akt-1*	luciferase	20°	18	47	4	
GR1310	*akt-1*	nrde-1	20°	19	52	6	0.0965

GR1310	*akt-1*	wago-1	20°	16	40	8	<0.0001
GR1310	*akt-1*	adr-1	20°	16	58	5	0.0410
GR1310	*akt-1*	set-25	20°	19	47	7	0.0345
GR1318	*pdk-1*	luciferase	20°	23	178	6	
GR1318	*pdk-1*	nrde-1	20°	20	161	23	<0.0001
GR1318	*pdk-1*	wago-1	20°	21	174	10	<0.0001
GR1318	*pdk-1*	adr-1	20°	20	144	7	<0.0001
GR1318	*pdk-1*	set-25	20°	23	170	4	0.0344
CB1370	*daf-2*	luciferase	15° until L2 & transferred to 25°C	31	308	9	
CB1370	*daf-2*	nrde-1	15° until L2 & transferred to 25°C	18	156	8	<0.0001
CB1370	*daf-2*	wago-1	15° until L2 & transferred to 25°C	20	134	4	<0.0001
CB1370	*daf-2*	adr-1	15° until L2 & transferred to 25°C	17	133	8	<0.0001
CB1370	*daf-2*	set-25	15° until L2 & transferred to 25°C	23	220	10	<0.0001
MAM71	DAF-16::GFP	luciferase	20°	18	92	5	
MAM71	DAF-16::GFP	nrde-1	20°	16	86	2	<0.0001
MAM71	DAF-16::GFP	wago-1	20°	16	92	8	<0.0001
MAM71	DAF-16::GFP	adr-1	20°	18	88	11	0.0065
MAM71	DAF-16::GFP	set-25	20°	18	80	14	0.2075

Table: S1

Strain	Allele	RNAi	Temperature (°C)	Median lifespan (days)	N	censored	P value vs control
N2		luciferase	20°	17	103	5	
N2		nrde-1	20°	14	87	6	<0.0001
N2		nrde-2	20°	17	106	4	
N2		nrde-3	20°	16	95	3	0.0187
N2		nrde-4	20°	15	116	1	<0.0001
CB1370	*daf-2*	luciferase	15° until L2 & transferred to 25°C	31	140	6	
CB1370	*daf-2*	nrde-1	15° until L2 & transferred to 25°C	17	125	8	<0.0001
CB1370	*daf-2*	nrde-2	15° until L2 & transferred to 25°C	22	137	6	<0.0001

CB1370	*daf-2*	nrde-3	15° until L2 & transferred to 25°C	21	140	6	<0.0001
CB1370	*daf-2*	nrde-4	15° until L2 & transferred to 25°C	20	124	4	<0.0001

Table: S2

ANEXO I

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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Dissertação de Mestrado, intitulada "***Uncovering the new regulators of DAF- 16/FOXO expression***", desenvolvida no Programa de Pós-Graduação em Genética e Biologia Molecular do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

Assinatura:_____
Nome da aluna: Sweta Sarmah

Assinatura:_____
Nome do orientador: Marcelo Alves da Silva Mori

Data: 25/03/2020

ANEXO II

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Data: 25/03/2020